Genetic analysis, QTL mapping and gene expression analysis of key visual quality traits affecting the market value of field pea

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Lasantha Chandana Ubayasena

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ABSTRACT

Visual quality is one of the major factors that determine the market value of field pea (*Pisum sativum* L.). Breeding for improved visual quality of pea seeds is currently a challenging task, because of the complexity and lack of sound genetic knowledge of the traits. The objectives of this research were to characterize the genetic basis and identify the genomic regions associated with four key visual quality traits (cotyledon bleaching in green pea, greenness in yellow pea, and seed shape and seed dimpling in both green and yellow types) in field pea. Biochemical and gene expression profiling to understand the molecular basis of post-harvest cotyledon bleaching in green pea was also addressed. Two $F_{5:6}$ recombinant inbred line (RIL) populations (90 lines from Orb X CDC Striker cross, and 120 lines from Alfetta X CDC Bronco cross) were developed and evaluated for visual quality traits in two locations in Saskatchewan, Canada in 2006 and 2007. The four quality traits evaluated all displayed a continuous range of expression with moderate to high heritability. Two genetic linkage maps utilizing 224 markers (29 simple sequence repeat (SSR) (from Agrogene) and 195 amplified fragment length polymorphism (AFLP)) and 223 markers (27 SSR and 196 AFLP) were constructed for the Orb X CDC Striker population and the Alfetta X CDC Bronco population, respectively. Multiple quantitative traits (QTL) mapping detected major QTLs on linkage group (LG) IV and LG V, as well as location- and year-specific QTLs on LG II and LG III associated with green cotyledon bleaching resistance. Nine QTLs controlling yellow seed lightness, three for yellow seed greenness, 15 for seed shape and nine for seed dimpling were detected. Among them, 5 QTLs located on LG II, LG IV and LG VII were consistent in at least two environments. The QTLs and their associated markers will be useful tools to assist pea breeding programs attempting to pyramid positive alleles for the traits. The bleaching resistant cultivar CDC Striker had a slower rate of chlorophyll degradation in cotyledons and a higher carotenoid to chlorophyll ratio in seed coats than the bleaching susceptible cultivar Orb when seed samples were exposed to high intensity light. An oligo-nucleotide microarray (Ps6kOLI1) was utilized to investigate the gene expression profiles of CDC Striker and Orb seed coats at different developmental stages. It clearly indicated that the expression of genes involved in the production and accumulation of

secondary metabolites was significantly different between these cultivars. The results of both biochemical and gene expression studies suggested the bleaching resistance in CDC Striker was not due to the accumulation of chlorophyll pigments in the cotyledons, but rather due to the ability of seed coats to protect them from photooxidation. Accumulation of specific carotenoids which could bind with the reaction center protein complex more effectively and accumulation of phenolic secondary metabolites which could enhance the antioxidant properties and structural integrity of the seed coats may lead to the bleaching resistant phenotype. Therefore, breeding green pea cultivars with higher seed coat antioxidant properties would improve both visual and nutritional quality. This research has provided several insights into molecular approaches to improve field pea visual quality for food markets.

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LIST OF ABBREVIATIONS

ABA : abscisic acid

ADP : adenosine diphosphate

AFLP : amplified fragment length polymorphism

ANOVA : analysis of varances

BDSa : bleached dehulled seed Hunter Lab “a” value

BDSb : bleached dehulled seed Hunter Lab “b” value

BDSL : bleached dehulled seed Hunter Lab “L” value

BWSa : bleached whole seed Hunter Lab “a” value

BWSb : bleached whole seed Hunter Lab “b” value

BWSL : bleached whole seed Hunter Lab “L” value

cDNA : complementary deoxyribose nucleic acid

Chl-a : chlorophyll-a

Chl-b : chlorophyll-b

cM : centiMorgans

CV : coefficient of variation

DAB : days after exposure to accelerated bleaching conditions

DAF : days after flowering

DBSa : dehulled bleached seed Hunter Lab “a” value

DBSb : dehulled bleached seed Hunter Lab “b” value

DBSL : dehulled bleached seed Hunter Lab “L” value

DD-PCR : differential display- PCR

DNA : deoxyribose nucleci acid

DT : desiccation tolerance

EST : expressed sequence tags

$F_{5:6}$: F_5 derived F_6 generation

GLIP : European Union Grain Legumes Integrated Project

GL-TTP : Grain Legumes Technology Transfer Platform

H^2 : broad-sense heritability

KEGG : Kyoto encyclopedia of genes and genomes

LG : linkage group

LOD : logarithm of the odds ratio

MQM : multiple QTL mapping

mRNA : messanger ribonucleic acid

NS : not significant

PCR : polymerase chain reaction

QTLs : quantitative trait loci

RAPD : random amplified polymorphic DNA

RFLP : restriction fragment length polymorphism

RIL : recombinant inbred line

RT-PCR: reverse transcription PCR

SAGE : serial analysis of gene expression

SD : standard deviation

SNPs: single nucleotide polymorphisms

SSR : simple sequence repeat polymorphism

STS : sequence tagged sites

UBDSa : unbleached dehulled seed Hunter Lab “a” value

UBDSb : unbleached dehulled seed Hunter Lab “b” value

UBDSL : unbleached dehulled seed Hunter Lab “L” value

UBWSa : unbleached whole seed Hunter Lab “a” value

UBWSb : unbleached whole seed Hunter Lab “b” value

UBWSL : unbleached whole seed Hunter Lab “L” value

WSa : whole seed greeness

WSL : whole seed lightness

σ^2_e : error variance

$\sigma^2_{G:}$ genotypic variance

$\sigma^2_{GL:}$ genotypic X location interaction variance

$\sigma^2_{GLY:}$ genotypic X location X year interaction variance

σ^2_{GY} : genotypic X year interaction variance

σ^2_P : phenotypic variance

CHAPTER 1

1. Introduction

Field pea (*Pisum sativum* L.) has been accepted throughout the world as a rich source of vegetable proteins and carbohydrates for human diets, as well as in animal feed formulations. In 2007, Canada accounted for 32% (3,379,400 MT) of the world total pea production (FAOSTAT data, 2009). Pea production from Saskatchewan contributed 74% of the Canadian pea production, followed by Alberta at 21%, and Manitoba at 5% (Statistics Canada, Field Crop Reporting Series, Vol.32, No. 5). The end use and the market value of the pea crop is highly dependant on visual traits of the seeds.

Based on the official grain grading guide of the Canadian Grain Commission (2008), acceptable natural color of pea seeds is considered one of the key quality factors determining grade. To qualify for the highest grade of green pea (Canada No. 1), seeds should have a natural green color with less than 2% bleached seeds (seeds with more than one-eighth of the surface of the cotyledon bleached to a yellowish color). For yellow pea, natural yellow color with less than 1% of other cotyledon color, such as green or orange, is the key to qualifying for the highest market grade (Canada No.1). Other than seed color, seed shape (round, as opposed to blocky or angular shape) and seed coat texture (smooth, as opposed to dimpled, or "golf –ball" seed surface) are often considered by pulse crop traders beyond the Canadian Grain Commission grading system for both green and yellow peas. In addition, seed size and uniformity of the seeds also play an important role in field pea trading.

Four QTLs controlling seed weight in pea were identified by linkage mapping, bulked segregant analysis and selective genotyping using RILs derived from two crosses (Timmerman-Vaughan et al. 1996). Biochemical changes during development of the seed pigments (chlorophyll a and b, violaxanthin, neoxanthin, β-carotene and lutein) in pea and genetic linkage analysis of the green seed color were assessed by McCallum et al. (1997). Significant differences in pigment accumulation and rate of degradation during seed development and seed maturation

between the parental lines (OSU442-15 X Primo) were observed. Four genomic regions controlling green seed color were reported by interval mapping using a linkage map produced from 199 molecular markers, bulked segregant analysis and selective genotyping. McCallum et al. (1997) studied the genetic control of green seed color and bleaching during seed development, in contrast to post-harvest bleaching under investigation in this study. The environmental effects on pea seed color and retention of green color have not been properly addressed to date. Involvement of at least three genes affecting seed coat and cotyledon color in pea genotypes and cotyledon bleaching resistance were reported (Lamprecht 1959; Dribnenki 1979), but no molecular markers linked to the retention of green seed color have been developed so far to facilitate pea breeding programs.

Chloroplast photosynthetic pigments of the cotyledon such as chlorophylls, carotenoids and xanthophylls have been reported as the pigments responsible for the green color of pea seeds (Steet and Tong 1996; Edelenbos et al. 2001). Bleaching of green seeds during storage is an external symptom of the intracellular break down of photosynthetic pigments as a result of long term exposure to bright light. The degradation of chlorophyll by photooxidation has been investigated in several plant species (Feierabend and Schubert 1978; Sagar et al. 1988; Eckhardt et al. 2004). Carotenoids have an important role in protecting chlorophyll pigments from bleaching (Griffiths et al. 1955; Anderson and Robertson 1960).

Dimpling of seeds, i.e., small, shallow impressions on the testa, is also a key visual quality trait in pea that determines market value. Mechanical and textural characteristics of the testa are the major determinant of the appearance of the seed surface. Pectic polysaccharide domains in cells and tissues of the testa play an important role in maintaining the mechanical properties of developing pea seeds, especially at the later stages of seed development (McCartney and Knox 2002). Involvement of a single gene, *mifo*, controlling the dimpling trait of pea seeds was reported by Lamprecht (1962); however, no environmental effects were assessed, nor were user-friendly markers developed. In addition, the genetics of seed shape and greenness in yellow cotyledon pea have not been fully characterized. Therefore, the current research was undertaken to study the genetic, genomic and biochemical characterization of several visual quality traits including cotyledon bleaching resistance in green pea, seed color in yellow pea and seed shape and seed dimpling in both green and yellow pea types.

The benefits to breeding programs include knowledge of the biochemical control, genetic control and environmental effects of key traits associated with visual quality in field pea and the identification of molecular markers linked with the genes controlling these traits. Since genetic markers are not affected by environmental conditions, markers will help breeders maintain the improved quality traits in breeding populations without the difficulties imposed by the need to select under erratic environmental conditions.

CHAPTER 2

2. Literature review

2.1 Pea production

Field pea plays an important role in human nutrition and an ingredient in animal feed formulations as a rich source of energy, fiber and most importantly protein (FAO 2008). Despite price fluctuations, cultivation of pea in western Canada has become more and more established in the past 20 years. This is mainly due to its ability to fix nitrogen and the suitability of pea as one of the main alternative crops to wheat barley and other grains (Bowren and Cooke 1975). Due to the cool climate, which is favorable for natural control of insects and diseases, and provides optimum conditions for pea growth and production, western Canada has become the world's largest producer of high quality peas.

Total dry pea production in Canada during the 2007 and 2008 cropping years reached 2.9 MT, which was about 70% of the total pulse production and also includes lentil, chickpea and dry bean (Statistics Canada, 2009). More than 80% of the Canadian pea production consisted of the yellow cotyledon type, 18% was green cotyledon type, and the remaining 2% was minor types including maple, marrowfat and Austrian winter (Statistics Canada, 2009).

2.2 Market quality

The market value of pulse crops is often determined by culinary properties which influence consumer and processor acceptance (Hosfield 1991; Nleya et al. 2000). Nleya et al. (2000) reported that important culinary traits of pulse crops are mostly associated with the visual characteristics of the seeds such as size, shape, seed coat color, cotyledon color, as well as seed uniformity and purity (Appendix 1). The nutritional properties of pulses such as protein concentration, protein quality and anti-nutritional factors (Nleya et al. 2000) were not considered a major factor in determining market value (Slinkard et al. 2000).

Determination of the market grades of pulse crops is mainly dependent on the visual quality characteristics of the dried seeds (Hosfield 1991; Slinkard et al. 2000; Canadian Grain

Commission 2008). In field pea, good natural color of the seeds is considered one of the key factors determining grade (Canadian Grain Commission 2008). To qualify for the highest human consumption market grade of Canada No. 1, a green pea sample should have a natural green color with less than 2% bleached seeds (seeds with more than one-eighth of the surface of the cotyledon changed to yellow). For yellow pea, natural yellow color with less than 1% of other color cotyledons is the key qualifying determinant for Canada No. 1. Other than seed color, seed shape (round, as opposed to blocky or angular shape), seed surface (smooth, as opposed to wrinkled or dimpled) and seed size are all considered in market grade determination.

Genetic improvement of pea seed quality at harvest and post-harvest stages has been identified as an important breeding objective (Ambrose 2008). Proper understanding of seed development is vital to genetically improve traits associated with visual and nutritional quality parameters as these factors often depend on the stage of seeds harvested (Ambrose 2008).

2.3 Seed development in pea

2.3.1 Structural changes

Pea seed development occurs inside the growing pea fruit which consists of a fertilized ovary and the outer pericarp (ovary wall). The pericarp protects the developing seeds while creating an optimum micro-climate by acting as a physiological buffer to the incoming nutrient flux to the seeds (Müntz et al. 1978). Seed development in pulse crops has been broadly identified as progressive events of embryogenesis which include cell division, cell expansion and seed desiccation (Munier-Jolain and Ney 1998a; Domoney et al. 2006). This process from fertilization to seed maturity includes maternal tissues and filial organs such as embryo and endosperm in a highly organized system (Borisjuk et al. 2004).

During the cell division phase, a single cell zygote becomes an embryo which consists of highly differentiated tissues and is mainly regulated by the filial genotype (Domoney et al. 2006). No significant dry matter accumulation has been reported during this stage and differentiation of these dividing cell clusters into highly specialized storage organs as well as other important filial organs follows at the end of this cell division phase (Ney et al. 1993). Borisjuk et al. (2005) described seven stages (stage I to stage VII) during this phase using histo-differentiation in *V. faba* embryos. They described seed development in two broad physiological

states, organogenesis and morphogenesis (Stage I to Stage III) and cotyledon development (Stage IV to Stage VII). The developing cotyledon tissues are mitotically very active at stage IV and the transition from the cell division phase to cell expansion starts at stage V (Borisjuk et al. 2005). In parallel with the cell division stage of embryogenesis, the legume fruit continues its development mainly by cell expansion in order to reach its final size (Carlson 1973). The transition of cell division to cell expansion in developing cotyledons of *Pisum* is a gradual change from the inner layers of the young cotyledon tissues to the outer abaxial regions (Smith 1973; Craig et al. 1979; Ambrose et al. 1987). The cotyledon cells continue expansion by accumulating storage products throughout stage VI finally reaching physiological maturity at stage VII (Borisjuk et al. 2005).

During seed development, the seed coat of pulses plays an important role in protecting and releasing nutrients to the growing embryo (Boeswinkel and Bouman 1995). The anatomical structure of seed coats of most of these crops consists of parenchyma cells with a layer of sclerenchyma and a vascular system (Van Dongen et al. 2003).

2.3.2 Physiological regulation of seed development

The cell division stage is much more important with respect to the seed growth potential than the seed filling stage (Munier-Jolain et al. 1998b). Seed growth rate of legumes is one of the major determinants of seed size and is poorly correlated with the duration of seed filling (Pfeiffer and Egli 1988). Munier-Jolain and Salon (2003) demonstrated that the sucrose influx to seeds during the cell division phase is the main factor affecting the mitotic activity of the pea seed embryo and not the incoming nitrogen flux. The importance of regulating this early stage mitotic activity at different reproductive nodes, where the seeds at upper reproductive nodes have a shorter duration for cell division compared to the lower reproductive nodes, has been studied intensively in an attempt to keep seed size uniform (Ney et al. 1993; Sagan et al. 1993; Ney and Turc 1993; Munier-Jolain and Ney 1998b). The role of plant growth regulators, including gibberellins, ABA, auxins and cytokinins, in regulating the rate of cell division during embryogenesis, has been evaluated (Rock and Quatrano 1995; Ozga et al. 2002; Quesnelle and Emery 2007). Cytokinins play an important role during seed growth by promoting embryogenesis and thereby increasing sink strength (Quesnelle and Emery 2007). Cytokinins are responsible for strengthening the sink capacity of the developing seeds by promoting cell

division and regulating sucrose metabolism (Brenner and Cheikh 1995; Emery and Atkins 2006). Borisjuk et al. (2002) demonstrated that legume embryogenesis and cotyledon differentiation is regulated metabolically and genetically by an intra-cotyledonary sucrose gradient. Seeds cease filling when they reach their maximum potential size as determined by cell number when photosynthetic assimilates are not limited (Munier-Jolain et al. 1998; Domoney et al. 2006). Seed dry weight is highly correlated with duration of the seed filling stage (Munier-Jolain et al. 1998b).

2.3.3 Genetic control of seed development

Understanding the genetic control of seed development is the key element to manipulate seed yield and quality attributes in crop plants. Mutated lines have been utilized intensively to study the genetics of seed development in pea (Blixt 1962; Murfet and Reid 1993; Cernac and Benning 2004). A phenotype of shrunken cotyledons (wrinkled) on pea seeds has been identified and characterized. These lines contain two alleles which alter the starch composition by changing the activity of starch branching enzyme I (SBE I) and ADP glucose pyrophosphorylase (Smith 1973; Hedley et al. 1986; Wang et al. 1990; Bhattacharyya et al. 1993). Transcription of sucrose synthase and ADP-glucose pyrophosphorylase are regulated by the level of sucrose in the developing cotyledonary cell layers (Borisjuk et al. 2002). Johnson et al. (1994), using pea seeds mutated by ethyl methane sulphonate (EMS), demonstrated the involvement of three loci in cell differentiation during seed development. Cernac and Benning (2004) identified a transcription factor regulating lipid and carbohydrate metabolism during seed development, WRINKLED 1 in *Arabidopsis*, which alters the storage product composition in the embryo. Alterations in seed storage composition during seed development could affect seed morphology (Domoney et al. 2006).

Two main phases of embryo development are the initiation phase of growth and the termination of growth when the embryo fills the seed sac (Raz et al. 2001). The first phase of embryo growth is regulated by FUS3/LEC type genes and the later stage of embryo growth, which mainly consists of accruing the embryo dormancy, is regulated by AB13 abscisic acid.

Radchuk et al. (2006) reported that SUCROSE NONFERMENTING-1 (Snf1)-related protein kinases (SnRK1) act as mediators of abscisic acid (ABA) during pea seed maturation. Repression of the SnRK1 gene results in maturation defects in pea, such as reduced conversion

of sucrose into storage products, lower globulin content, altered cotyledon surface, shape, and symmetry, as well as occasional precocious germination. SnRK1 also repressed some genes involved in regulation and signaling of developmental events independent of ABA such as chromatin reorganization, cell wall synthesis, biosynthetic activity of plastids, and regulated proteolysis.

2.4 Genetic mapping and QTL analysis in plants

Most economically important crop traits are controlled by polygenes, i.e., exhibiting continuous phenotypic variation, and are often referred to as quantitative traits (Paterson et al. 1988; Paterson et al. 1990; Paterson et al. 1991; deVicente and Tanksley 1993; Tanksley 1993). These quantitative traits are controlled by many genes with relatively small contributions often modified by environmental factors. The genes or loci controlling quantitative traits have been described as quantitative traits loci (QTL).

The basic principle of mapping QTL was first discovered with the finding of an association of a complex quantitative trait with a simple monogenic trait by Sax (1923) and further developed to map such QTLs (Thoday 1961). Several methods have been reported to identify QTL regions based on marker linkage, including single marker QTL analysis (Weller 1986; Luo Kearsey 1989) and multiple marker QTL analysis based on maximum likelihood estimates (Jensen 1989; Lander and Botstein 1989; Knapp et al. 1990). The main disadvantage of the single marker QTL analysis is the decreasing power of detecting QTLs when markers are distant from the respective QTL. This has been overcome in interval mapping (IM) by considering both segregation information of two linked markers and maximum likelihood information simultaneously (Landon and Bostein 1989).

The most commonly used QTL mapping methods are IM (Lander and Botstein 1989) and multiple QTL model based methods (MQM) (Jansen 1993, 1994; Jansen and Stam 1994). In IM, the likelihood map of having or not having a segregating QTL at each position in the genome is calculated based on the marker segregation information and phenotypic estimates of the mapping population. In IM a QTL is declared when the ratio of these two likelihoods statistics (LOD) exceeds the significant threshold level. The number of individuals in the mapping population and the size of the genotypic effect of the QTL greatly influence the accuracy of detecting QTLs using IM (Van Ooijen 1992). QTLs with larger genotypic effects were detected and located more

precisely compared to QTLs with smaller effctes where the detected QTL intervals were highly variable. Jansen (1994) reported that the probability of Type I (*ie*., detecting a false positive QTLs) and Type II (*ie*., not detecting a QTL) errors is much higher in IM compared to alternative approaches such as simultaneous mapping of multiple QTLs. In addition, interfering effect of QTLs located elsewhere in the genome on the QTL of interest are not considered in IM (Janson 1993).

The MQM method described by Jansen (1994) and Jansen and Stem (1994) overcame the defects of the IM approach by reducing Type 1 and Type II errors. The MQM approach eliminates the "noise" contributed from nearby QTLs by implementing a two-stage QTL mapping procedure, where the first stage identifies the important markers linked with the QTLs by multiple regression, and the second stage by eliminating the noise contributed by nearby QTLs using neighbouring markers as co-factor markers. The MQM model uses a combination of interval mapping and multiple regressions methods (Jansen 1992). QTL mapping using MQM mapping is not only a powerfulmethod to detect QTLs but also a useful tool to identify underlying genotype by environment interaction (Jansen et al., 1995).

The concept of utilizing the association between simple monogenic traits inherited by simple Mendelian principles to map QTLs of complex traits has been revolutionized with the development of DNA based molecular markers which have a monogenic mode of inheritance with no environmental influence (Phillips and Vasil 1994). Saturated genetic linkage maps utilizing molecular markers have been used extensively to study quantitatively inherited agronomically important traits in many crops (Paterson et al. 1988; Tanksley 1993). In addition to the molecular marker coverage of the genome, the genetic structure and the size of the mapping populations affect the resolution of QTLs (Paterson et al. 1988; Paterson et al. 1990; Paterson et al. 1991). QTL analysis is one of the most effective approaches of studying genetically complicated traits in plants such as biotic and abiotic stress resistances, yield components, and seed quality (Mansur et al. 1996; Young 1996; Orf et al. 1999; Del et al. 2003; Hyten et al. 2004; Miyamoto et al. 2004; Tar'an et al. 2004; Timmerman-Vaughan et al. 2004).

2.4.1 Molecular markers for linkage mapping

Identification of QTLs controlling fruit size, pH, and total soluble solids of tomato using molecular markers (Paterson et al. 1988) was one of the first reports describing the potential of

genetic linkage maps based on molecular markers, in this case Restriction Fragment Length Polymorphism (RFLP), to resolve quantitative traits into discrete Mendelian factors. Since then RFLP markers have been utilized to develop genetic linkage maps in many organisms (Landry et al. 1991; Landry et al. 1992; Ajmone Marsan et al. 2001; Cogan et al. 2005).

With the development of PCR (Polymerase Chain Reaction), which can amplify target DNA sequences using random or specific short oligonucleotide primers and detection of the amplified regions on agarose or polyaccrylamide gels, the practical difficulties of RFLP have been overcome. These include requirement of a large amount of high quality DNA, use of radioisotopes, time consuming and complex analytical techniques. Therefore, PCR has become the technique of choice in molecular linkage mapping projects (Saiki et al. 1988; Arnheim et al. 1991; Erlich et al. 1991). The most popular PCR-based molecular markers for linkage mapping, which replaced RFLP, were RAPD (Random Amplified Polymorphic DNA) (Williams et al. 1990), STS (Sequence Tagged Sites) (Olson et al. 1989), Simple Sequence Repeat polymorphism (microsatellite or SSR) (Tautz 1989; Weber and May 1989; Hyten et al. 2004; Schmalenbach et al. 2008; Xu et al. 2008; Jegadeesan et al. 2010) and Amplified Fragment Length polymorphism (AFLP) (Vos et al. 1995; Powell et al. 1997; Eujayl et al. 1998; Lei et al. 2007).

However, with the recent development of DNA sequencing techniques like next-generation sequencing platforms and advanced analytical techniques which enable more and more multiplexing and high-throughput analysis with automation facilities, investigation, identification and utilization of the most abundant genomic sequence polymorphism, i.e., SNPs (Single Nucleotide Polymorphisms) based PCR assays have revolutionized genetic linkage mapping in plants (Sato and Takeda 2009; Yang et al. 2010).

2.5 Genetic composition, linkage mapping and QTL analysis in pea

2.5.1 Genetic composition of pea

Taxonomically, field pea (*Pisum sativum*) belongs to the family Fabaceae, sub-family Papillionaceae, tribe Vicieae, genus *Pisum*, species *sativum* and subspecies *sativum* (Ben Ze'en and Zohary 1973; McPhee 2007). Several progenitors of *P. sativum* have been identified, i.e., *P. s* ssp. *elatius, P. s* ssp. *humile, P. s* ssp. *arvense, P. s* ssp. *hortense,* and their utilization in pea

breeding is discussed by Muehlbauer (1992). The genome size of pea is 4800 Mbp arranged in 7 chromosomes (2n=2x=14) (Bennett and Leitch 2005; Zonneveld et al. 2005). The structure of each chromosome has been well documented including its relative length, centromereric location, secondary constructions, presence of satellites and other chromosomal rearrangements (Blixt 1958; Ben Ze'en and Zohary 1973; McPhee 2007; Hall et al. 1997a; Hall et al. 1997b).

2.5.2 Genetic linkage maps of pea

The first genetic linkage map of pea with seven linkage groups (LG) was developed by Lamprecht (1948, 1958) utilizing morphological marker segregation in several populations derived by two-point crosses. Blixt (1972) later assigned these morphological mutations to seven linkage groups. These seven LG have been assigned to their respective pea chromosomes using standard karyotype and a set of translocation lines (Blixt 1958, 1959). The beginning of global pea map rearrangements started with the introduction of isozyme variations and with the incorporation of more morphological markers to construct genetic linkage maps (Weeden and Marx 1984; Mahmoud et al. 1984; Weeden and Marx 1987). Construction of pea genetic linkage maps has been enhanced with the introduction of molecular markers. The first molecular marker based pea map with increased marker density and well defined LG was published by Ellis et al. (1992) followed by Timmerman et al. (1993) and Weeden et al. (1993).

The first PCR based pea genetic linkage map was constructed using RAPD markers and resulted in the identification of a gene conferring resistance to pea enation mosaic virus (Yu et al. 1995). A detailed pea genetic map using a combination of allozyme, RFLP and RAPD markers, and location of *er-1*, a recessive gene conditioning resistance to powdery mildew, was reported by Timmerman et al. (1994). The marker density of pea genetic linkage maps was significantly increased with the utilization of AFLP (Timmerman-Vaughan et al. 1996; Tar'an et al. 2003; Tar'an et al. 2004) and SSR markers (Loridon et al. 2005). A detailed review of pea genetic mapping was recently published (McPhee 2007).

Current developments in plant genomics research allow for the construction of functional genetic maps using polymorphisms detected within the genes encoding enzymes involved in primary metabolism. Aubert et al. (2006) developed a functional genetic map spanning 1458 cM utilizing 363 polymorphic markers located in gene sequences and described the utility of this map in identifying candidate genes in pea.

2.5.3 QTLs identified in pea

Compared to other economically important food crops, relatively few QTL mapping studies have been reported in pea (Swiecicki and Timmerman-Vaughan 2005; McPhee 2007). Timmerman-Vaughan et al. (1996) reported the first QTL analysis in pea using a genetic linkage map of two populations segregating for seed weight (F_2 progenies and single seed descent recombinant inbred lines (RILs)) using 101 RFLP, 58 RAPD and 40 AFLP markers. Despite the identification of several QTLs associated with seed weight from two populations, only one QTL located on linkage group III was identified in both populations. One of the major QTLs identified on LG III was mapped to orthologous regions responsible for control of seed weight in *Vigna* spp. (Fatokun et al. 1992) and soybean (Maughan et al. 1996).

Tar'an et al. (2003; 2004) constructed genetic linkage maps based on AFLP, RAPD and STS markers and reported QTLs associated with lodging resistance (QTLs on LG III and VI), plant height (QTLs on LG III and two on unassigned LGs C and D), mycosphaerella blight (QTLs on LG II, IV and VI), grain yield (QTLs on LG II, VI and VII), seed protein concentration (QTLs on LG III, VI and unassigned LG A), and maturity (QTLs on LG II, III and VI). Genetic control of green seed color in field pea and associated QTLs was studied using F_2 individuals and F_2 derived F_3- family populations by McCallum et al. (1997). Several QTLs associated with seed color on LG III, IV, V and VII were reported. Moreover, additional QTLs on LG II, III and VII associated with the color space U and V chrominance were reported. Dirlewanger et al. (1994) discovered QTLs associated with node number (3), earliness (2), plant height (1) and resistance to *Ascochyta* blight (1). Prioul et al. (2004), using a linkage map based on RAPD, SSR and STS marker polymorphism of a RIL population, identified 10 and 6 QTLs associated with *Ascochyta* blight resistance at the seedling stage and adult plant stages, respectively, and four more developmental stage independent QTLs. In addition to *Ascochyta* blight resistance, three QTLs for flowering date and plant height were reported.

Even though different research groups identified QTLs controlling the same phenotypic trait, in pea, these maps could not be compared due to lack of common markers (Swiecicki and Timmerman-Vaughan 2005; McPhee 2007). Several attempts have been reported to construct a consensus pea genetic linkage map with limited success (Weeden et al. 1998; McPhee 2007).

2.6 Biochemical basis of green pea bleaching

Classical genetic studies revealed that *I*, *pa*, *gla* and *vim* loci have major effects on conditioning chlorophyll retention of the cotyledons during seed maturity in green pea genotypes by altering senescence related chlorophyll degradation (Blixt 1962; Weeden and Wolko 1990). Bleaching in green pea has been biochemically described as the degradation of chlorophyll pigments from the green cotyledon tissues due to environmental factors during seed maturation (Maguire et al. 1973). Accelerated green cotyledon bleaching occurred when the seeds were exposed to light and the seed moisture content exceeded 20% (Riehle and Muelbauer 1975). Gubbels and Ali-Khan (1990) described a positive correlation between hard seeds and bleaching rate.

Edelenbos et al. (2001) studied the color and pigment composition of processed peas grown under two light regimes and detected 17 pigments including eight xanthophylls, four chlorophyll-b related compounds, four chlorophyll-a related compounds and one type of carotene. Furthermore, they found significant differences in pigment composition between cultivars and stages of seed maturity. McCallum et al. (1997) reported that the rate of chlorophyll degradation in the bleaching resistant pea cultivar 'Promo' was significantly lower than that of the bleaching susceptible cultivar 'OSU442-15'. Seed tissue analysis of both cultivars at maturity indicated a low chlorophyll a:b ratio in seed coats indicating lower chlorophyll stability. Despite studying the pigment composition of whole seeds, the pigment dynamics of seed coats and cotyledons during seed development, maturity and post harvest bleaching were not addressed by McCallum et al. (1997).

The dynamics of chlorophyll degradation and enzyme activity in seeds under simulated conditions has been reported in field peas by Cheng et al. (2004). Higher rates of chlorophyll degradation in cotyledons were observed for seeds soaked in water and exposed to light when compared to dry seeds exposed to light. A high activity level of chlorophyllase, one of the enzymes responsible for catabolism of chlorophyll (Matile et al. 1999), was found in soaked seeds for both bleaching resistant and susceptible pea cultivars, The differences detected for lipoxygenase and chlorophyll degrading peroxidase activity between soaked and dry seeds was not related with the chlorophyll loss. The activity of all three enzymes investigated did not change during the bleaching period with dry seeds. Furthermore, the biochemical properties of the seed coats during seed development and bleaching periods has not been addressed to date.

The process of light mediated chlorophyll degradation is known as photo-oxidation in plant tissues (Feierabend and Schubert 1978; Sagar et al. 1988; Niyogi 1999; Eckhardt et al. 2004). Exposure of photosynthetic plant tissues to excessive light could lead to the generation of highly reactive intermediates and byproducts which trigger photo-oxidation (Foyer et al. 1994a; Foyer et al. 1994b). Protection of the photosynthetic apparatus from photo-oxidation was reviewed by Niyogi (1999), who summarized four main mechanisms of photo-protection. Plants have adapted in many ways to protect the photo-oxidation process by dissipation of excess light energy as heat (Demmig-Adams 1990; Demmig-Adams and Adams 1992; Demmig-Adams et al. 1996), alternative electron transport pathways (Mehler 1951; Artus et al. 1986; Asada 1999), chloroplast antioxidant systems (Foyer et al. 1994a; Polle 1997) and repair of photosystem II (Aro et al. 1993). In addition, the presence of carotenoids play an important role in protecting chlorophyll pigments from bleaching caused by high light intensities (Griffiths et al. 1955; Anderson and Robertson 1960).

2.6.1 Photosynthesis in seeds

The chloroplasts present in seed embryonic tissues are photoheterotrophic in nature and differ in their structural and physiological role from plastids in leaf chloroplasts (Asokanthan et al. 1997). High photosystem II activity and high rate of uncoupled electron transport in the plastids of seeds were reported (Athwal et al. 1998). Asokanthan et al. (1997) reported that the primary role of this photosynthetic apparatus is not to photoassimilate CO_2 but to use the light reaction to produce ATP and NADPH to facilitate seed storage metabolism utilizing the assimilates supplied by maternal tissues. The additional role is to provide oxygen to the growing embryo to adapt the low oxygen availability within the seed which could promote higher respiratory and biosynthetic activity (Rolletschek et al. 2003).

2.6.2 Role of carotenoids and phenolics as antioxidents

The photosynthetic apparatus is highly efficient in capturing light to fix CO_2 under normal physiological conditions (Krieger-Lizkay 2005). However, if the energy is not efficiently used, extra light energy could be deleterious to the photosynthetic reaction center by producing energy excited chlorophyll triplet formation (Hoff 1979; Kirmaier and Holten 1987). Under aerobic conditions, these triplet chlorophyll molecules can form singlet oxygen, a powerful

oxidizing agent which is capable of damaging proteins, lipids and DNA (Krinsky 1971); this process is known as photooxidation. The production of singlet oxygen in photosynthetic tissues is responsible for most of the light induced inactivation of photosystem II, and distruction of phtosystem II by degrading D1 protein and pigments (Prasil et al. 1992; Aro et al. 1993).

These dangerous singlet oxygens can be quenched by carotenoids which are in close association with the photosynthetic apparatus (Edge and Truscott 1999). The carotenoids can exchange the triplet state from excited chlorophyll and later dissipate that energy as heat. β-carotene and other antioxidents deactivate singlet oxygen and thereby protect chlorophyll against photooxidation (Telfer 2002; Trebst 2003; Roszak et al. 2004).

Phenolic compounds naturally occurring in plant tissues, such as flavonoids (Decker 1997), chalcones and 3,4-dihydroxychalcones (Dziedzic and Hudson 1983), hydroxycinnamic acid (Natella at el. 1999), and proanthocyanidins (Hatano et al. 2002) have high antioxidant acitivity through scavenging free radical oxygens. Dueñas et al. (2006) reported that the seed coats of legumes tend to have higher antioxidant properties than the cotyledons due to the presence of phenolic compounds with flavonoid structures.

2.7 Transcription profiling to identify genes responsible for trait expression in plants

Identification and characterization of genes responsible for economically important phenotypic traits in agricultural crop species and their interactions allow breeders to manipulate traits of interest by pyramiding gene combinations through molecular marker assisted selection (Flavell 2010). High throughput transcription profile analysis has been extensively used in identification and deciphering gene coordination in controlling economically important traits under various environmental conditions (Venter and Botha 2004). Genes expressed in different tissues, genotypes, developmental stages or under different growth conditions could be qualitatively and quantitatively determined by comparing the concentration of respective mRNA of the tissue (Kuhn 2001). Recent developments in high throughput gene expression technologies, such as cDNA microarrays (Schena et al. 1995; Hughes et al. 2001), cDNA-AFLP (Amplified Fragment Length Polymorphism) (Vos et al. 1995), SAGE (Serial Analysis of Gene Expression) (Velculescu et al. 1995), and DD-PCR (Differential Display- PCR) (Liang and Pardee 1992) enable researchers to unveil genome-wide, multi-gene expression patterns. Among these techniques, cDNA-AFLP and cDNA microarrays have been extensively used in profiling

transcriptomes (Kuhn 2001; Donson et al. 2002). Donson et al. (2002) claimed that both cDNA-AFLP and cDNA microarrays have significant value in functional genomic analysis, genome annotation and identification of gene regulatory elements. The use of cDNA microarrays enable researchers to study global gene expression patterns of different biological tissue types under a variety of experimental conditions accommodating large numbers of cDNA spots in one microarray and the ability to hybridize two cDNA samples under investigation in parallel (Desprez et al. 1998; Schena et al. 1995; Kuhn 2001; Donson et al. 2002;). With the recent development of transcriptome analysis flat forms, such as cDNA-AFLP, microarrays, macroarrays, and gene chips, the study of gene expression profiles of plant tissues at different developmental stages including seeds is possible (Schaffer et al. 2000; Weber et al. 2005; Gutierrez et al. 2006). Among the available forms of transcriptome analysis, microarrays are a useful tool due to recent advancements in array printing technologies allowing increased precision, improved protocols and availability of well designed, user-friendly software to analyze large amounts of data to draw precise scientific conclusions (Hegde et al. 2000; Saeed et al. 2003).

Despite the many applications of cDNA microarrays in transcription profiling, there have been some inherent limitations such as cross-hybridization of homologous DNA, and low reproducibility of spotted DNA on the array (Donson et al. 2002; Alba et al. 2004; Meyers et al. 2004). Brazma et al. (2001) and Yang and Speed (2002) emphasized the need for biological and technical replication, maintenance of a high level of manufacturing quality control, and validation of cDNA microarray data by independent methods such as quantitative reverse transcription PCR (qRT-PCR), in order to enhance the biological relevance of transcription analysis based on cDNA microarray data.

2.7.1 Use of cDNA microarrays

Schena et al. (1995) developed and utilized the first cDNA microarray using 48 duplicated cDNA spots of *Arabidopsis thaliana*, to understand gene expression differences between shoots and roots using simultaneous hybridization of two cDNA pools from respective tissues labeled with fluoresein and lissamine. They recognized 26 genes differentially expressed with more than a 5-fold difference between roots and shoots. They reported a 500-fold and 60-fold difference in the expression of the light regulated CABI gene and a transformed HAT4

transcription factor of a transformed plant, respectively. Furthermore, transcription profiling with cDNA microarrays consisting of 1443 *Arabidopsis thaliana* genes was used to investigate different organs at different developmental stages (Ruan et al. 1998). They reported a high level of transcription complexity in different organs by observing the level and the number of genes up and down regulated in different tissues. The most differentially expressed genes between roots and leaves were involved in photosynthesis. Most of the genes up regulated in leaves were down regulated in flowers; however, a different set of 200 genes were up regulated in flowers compared to leaves.

A dynamic pattern of transcript accumulation was reported during the transition of key developmental stages (3-7, 7-14 and 21-28 days post anthesis) in wheat caryopses (Laudencia-Chingcuanco et al. 2007). The functional annotation of the genes with high levels of expression, at each developmental time-point, was highly associated with the respective developmental events of the wheat caryopsis. The cDNA microarray used in this study consisted of 7835 genes. Identification and regulation of symbiotically activated genes during two different endosymbioses with soil prokaryotes (nitrogen-fixation nodulation) and soil fungi (arbuscular mycorrhiza) using *in silico* and *Medicago truncatula* cDNA microarray based transcriptome profiling was reviewed by Küster et al. (2007). About 100 genes were co-induced during nodulation and arbuscular mycorrhizal infection which were associated with symbiotic efficiency (El Yahyaoui et al. 2004; Küster et al. 2004; Manthey et al. 2004; Domoney et al. 2006).

2.7.2 Transcription profiling during seed development using cDNA microarray

Several transcription profiling studies have been reported related to the developmental stages of flowers and early fruit (silique) and seed development in *Arabidopsis* (Girke et al. 2000; Ruuska et al. 2002; Becker et al. 2003; Honys and Twell 2003; Hennig et al. 2004; Wellmer et al. 2004). Involvement of MAD-S box gene expression during early flower development and during the organogenesis of whorls was reported using a flower specific cDNA microarray (De Bodt et al. 2003; Wellmer et al. 2004). Firnhaber et al. (2005) identified more than 700 genes of *Medicago truncatula* which are involved in development expression regulation in flowers and pods using a microarray which consisted of 6300 non-redundant genes using a time course expression profiling study. They highlighted the involvement of gene-encoded proteins with known functionality to seed metabolism, regulation and signaling.

A proteomic and transcriptome analysis to investigate the seed development of *M. truncatula* was carried out by Gallardo et al. (2007). They studied the transcription and protein profiles in isolated seed coats, endosperm and embryo and demonstrated that these two profiles were parallel for 50% of the comparisons. The divergent patterns were explained as post transcriptional modifications to the transcriptome. This study revealed that the main contribution made to the developing embryo by the surrounding seed tissues included compartmentalizing enzymes involved in methionine biosynthesis, which regulates the availability of sulfur containing amino acids for embryo protein synthesis, regulating the metabolic shift toward seed maturation by decreasing the level of metabolism of seed coat and endosperm tissues, and increasing proteases in seed coat and endosperm to provide a supplementary source of amino acids to the growing embryo at later stages of seed development. Furthermore, this study demonstrated a high level of expression of transporters involved in nutrient import and intra-seed translocations.

Buitink et al. (2006) reported regulatory processes and protective mechanisms leading to desiccation tolerance (DT) in seeds using cDNA microarrays consisting of 16,086 genes by monitoring the transcriptome of desiccation-sensitive 3-mm-long radicals of *M. truncatula* seeds. Transcription profiles of the embryos before and after the acquisition of DT during maturation indicated that more than 1300 genes were differentially expressed and majority were involved in carbon metabolism. They also observed that up-regulation of regulatory genes during drought stress in plants during normal seed maturation. Down-regulation of genes involved in cell cycle, biogenesis, primary and energy metabolism was also reported.

2.8 Objectives

The specific objectives of this study were as follows.

1. To characterize the genetic basis of cotyledon bleaching resistance in green pea, seed color in yellow pea, seed shape, and seed dimpling in both green and yellow pea types.

2. To identify the genomic regions (QTLs) associated with the control of cotyledon bleaching resistance in green pea, seed color in yellow pea, seed shape, and seed dimpling in both green and yellow pea types.

3. To characterize the genetic basis of cotyledon bleaching resistance in green pea, seed color in yellow pea, seed shape, and seed dimpling in both green and yellow pea types.

2.9 Hypotheses

1. Cotyledon bleaching resistance in green pea, seed color in yellow pea, seed shape, and seed dimpling in both green and yellow pea types are controlled by multiple genes and their expression is influenced by environmental factors.

2. The field pea RIL populations Orb X CDC Striker and Alfetta X CDC Bronco developed in this research will segregate for seed shape and seed dimpling, the Orb X CDC Striker population will segregate for cotyledon bleaching resistance, and the Alfetta X CDC Bronco population will segregate for seed color, and genetic maps can be constructed utilizing molecular marker linkage information. These visual quality traits are controlled by several regions of the pea genome which can be identified using linked molecular markers and phenotypic variability of the RILs.

3. Chemical and physical properties of the seed coat contribute to green cotyledon bleaching resistance, these properties are under genetic control, and can be characterised by gene expression differences in pea cultivars contrasting in bleaching resistance.

CHAPTER 3

3. Genetic control and QTL analysis of cotyledon bleaching resistance in green field pea (*Pisum sativum* L.)

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3.1 Abstract

Resistance to bleaching is an important factor for quality grading for Canadian green field pea and an important selection criterion in green pea improvement. This research was conducted to determine the genetic control of bleaching resistance in green peas using 90 recombinant inbred lines (RILs) derived from a cross between cultivars Orb and CDC Striker. These lines were evaluated under field conditions for two years in two locations in Saskatchewan, Canada. Harvested whole seeds and cotyledons were evaluated for greenness using the Hunter Lab colorimeter before and after exposure to a high light intensity accelerated bleaching treatment. The RILs were genotyped using amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) molecular markers. Heritability estimates for whole seed and cotyledon greenness were moderate (0.72 and 0.69, respectively), and increased when assessed after exposing whole seeds to accelerated bleaching conditions (0.83 and 0.82 for seed coat and cotyledons, respectively). The genetic linkage map constructed based on a total of 224 AFLP and SSR markers spanned over 890 cM of the pea genome. Multiple QTL mapping (MQM) detected major QTLs on LGIV and LGV, as well as location- and year-specific QTLs on LGII and LGIII associated with green cotyledon bleaching resistance in field pea. The results demonstrated the importance of the seed coat in protecting the cotyledons from bleaching.

Key words: field pea, bleaching, genetic mapping, amplified fragment length polymorphisms, simple sequence repeats, QTL mapping.

3.2 Introduction

Field pea (*Pisum sativum* L.) is a nutritious whole food consumed in many countries, which provides proteins, complex carbohydrates, and many essential micronutrients. In 2007, Canada accounted for 29% (3.0 million tonnes) of the world pea production (FAOSTAT, 2008). Two major market classes of field pea, yellow cotyledon and green cotyledon, are produced in Canada. The green cotyledon market class typically comprised approximately one-third of the total production (FAOSTAT, 2008). Based on the grain grading guide of the Canadian Grain Commission (2008), good natural color of seeds is considered one of the key quality factors determining the market values. To qualify for the highest market grade of green pea (Canada No. 1) seeds should have a natural green color with less than 2% bleached seeds, defined as seeds with more than one-eighth of the surface of the cotyledon bleached to a yellowish color. The down grading of pea from human consumption to feed markets could result in a loss of up to $100 or more per tonne (Statistics Canada 2009). Therefore, cotyledon bleaching during seed maturation or during seed storage periods is a crucial determinant of the farm gate value of green pea (Shepherd 1959; Holden 1965; McCallum et al. 1997; Cheng et al. 2004). Furthermore, bleaching has some adverse effects on seed germination and early seedling vigor in field pea (Maguire et al. 1973; Loria 1979).

The pigments responsible for green cotyledon color of pea seeds are the chloroplast photosynthetic pigments, including chlorophylls, carotenoids and xanthophylls (Steet and Tong 1996; Edelenbos et al. 2001). Degradation of chlorophyll from the cotyledons during seed maturation and senescence has been well characterized in legumes (Matile et al., 1999). Several genes associated with seed color, including *i*, *pa*, *gla* and *vim*, have been detected in green peas (Blixt, 1962; Weeden and Wolko, 1990). Bleaching due to environmental conditions during the maturation and post-harvest storage periods have been demonstrated as a different process from senescence-related chlorophyll loss (Matile et al., 1999; Cheng et al., 2004). The severity of bleaching is highly dependent on the environmental conditions during the seed maturation period prior to harvest (Riehle and Muelbauer 1975). Wet and dry cycles and exposure to light

enhanced bleaching. Hence the loss of green color due to light was described as photo-destruction of chlorophyll from the cotyledons (Holden 1965; Riehle and Muelbauer 1975). Cotyledon bleaching during storage is an external symptom of intracellular break down of photosynthetic pigments due to long term exposure to bright light. The degradation of chlorophyll by photooxidation has been demonstrated in several plant species (Feierabend and Schubert 1978; Sagar et al. 1988; Eckhardt et al. 2004).

Biochemical changes of the pigments (chlorophyll a and b, violaxanthin, neoxanthin, β-carotene and lutein) during the seed developmental stages, and genetic linkage analysis of green seed color were reported by McCallum et al. (1997). Significant differences in pigment accumulation and rate of breakdown during seed development and seed maturation between two pea cultivars (OSU442-15 and Primo) were observed. Genomic regions on linkage groups (LG) III, IV, V and VII affecting green seed color were reported by McCallum et al. (1997). Involvement of at least three genes affecting seed coat and cotyledon color in pea genotypes and cotyledon bleaching resistance were previously reported (Lamprecht 1959; Dribnenki 1979). No genotype x environment analysis or molecular markers were reported for the retention of green seed color in pea.

Improvement of bleaching resistance has been an objective of pea breeding world-wide. However, the lack of understand of the inheritance of this trait, accurate phenotypic characterization, and knowledge of the effects of the environment on the trait has hindered efforts to deliver improved cultivars. The objective of this research was to characterize the genetic basis of green cotyledon bleaching resistance and identification of the genomic regions (QTLs) associated with this trait in field pea.

3.3 Materials and Methods

3.3.1 Plant materials

A mapping population consisting of 90 recombinant inbred lines (RILs) was developed from the cross between *P. sativum* ‘Orb’ and *P. sativum* ‘CDC Striker’. CDC Striker is a green cotyledon cultivar with good bleaching resistance developed by the Crop Development Centre, University of Saskatchewan (Warkentin et al. 2004). Orb is a green cotyledon cultivar with poor

bleaching resistance developed by Sharpes International Seeds, UK. RILs were developed from 90 F_2 plants originating from a single F_1 plant. The hybridity of the F_1 plant was confirmed using SSR markers. These 90 F_2 plants were then advanced to $F_{5:6}$ generation by single seed decent and the bulked seeds were multiplied to form $F_{5:7}$ seeds in the greenhouse. The $F_{5:7}$ seeds of 90 RILs were used in the field experiments in 2006 and the seeds harvested from 2006 experiments ($F_{5:8}$) were used in 2007 field evaluations.

3.3.2 Evaluation of RILs for green cotyledon bleaching resistance

3.3.2.1 Field experiments

Field experiments to evaluate the 90 RILs along with the two parental cultivars were conducted in two locations in Saskatchewan in 2006 and 2007. The locations were Saskatoon, located in the Dark Brown soil zone, and Rosthern, located in the Black soil zone. Experiments were laid out in a 10 X 10 simple lattice design with two replicates and plot size of 1 m^2 (micro-plots). Individual micro-plots were hand-harvested when 95% of the pods reached maturity. The harvested seeds were cleaned and two representative subsamples of 50 g were drawn from each plot. These subsamples were used to characterize the green cotyledon bleaching resistance.

3.3.2.2 Assessment of green cotyledon bleaching resistance

Seed color was determined using a Hunter Lab colorimeter (Hunter Associates Lab Inc., Reston, Virginia, USA). The Hunter Lab colorimeter gives three-dimensional color estimates corresponding to the ganglion cells of the human eye with respect to the lightness (L), redness-greenness (a) and yellowness-blueness (b) of the sample (Marcus 1998). These Hunter Lab L, a, and b values have been used in many studies in evaluating the color differences in plants and food materials (Marshall et al. 1988; Ameny and Wilson 1997). Seeds were mixed and reoriented between scans and the Hunter Lab L, a, and b values of each sample were assessed by taking the average of three scans.

Color readings of the whole seeds as well as the cotyledons were recorded before and after exposure to the accelerated bleaching protocol described below. Seed coats were removed by splitting the seeds using a Satake Grain Testing Mill model TM05 (Satake Corporation, Taito-

ku, Tokyo, Japan). A detailed description of the different color measurement stages is given in Fig 3.1.

3.3.2.3 Accelerated bleaching conditions

A growth chamber equipped with both fluorescent and incandescent lights was utilized to accelerate bleaching of whole pea seeds and cotyledon samples. Seeds or cotyledon samples were placed in transparent clear plastic bags (Nasco Whirl-Pak, Fort Atkinson, WI, USA) with holes made by piercing with a 2 mm needle to facilitate air exchange. Light intensity was measured using a quantum meter (Model QMSS, Apogee instruments Inc. Logan, UT, USA) placed on the top surface of the sample bags. The height of the light hood was adjusted to deliver 1100 µmol $m^{-2}s^{-1}$ light intensity. The temperature and the relative humidity during the entire experiment were maintained at 23 ^{o}C and 60%, respectively. Seed bags were randomly spread over the bench after flattening to produce a single seed layer. Initially, samples were kept in the dark at 23 ^{o}C and 60% relative humidity for 48 hours within the chamber to achieve uniform moisture content. Moisture content of the seeds was assessed at 36 and 48 hours after exposure to the above conditions using control seed samples and it was determined that seed moisture content reached equilibrium at 12% (data not shown). After the equilibration period, samples were exposed to continuous 24 hour day light for 3 weeks for whole seeds and one week for cotyledons. Samples were mixed within the bags and bags were rearranged over the bench every 24 hours in order to minimize experimental error.

3.3.3 DNA extraction and molecular marker analysis

Pooled leaf samples from 10 plants per line (RILs and parental cultivars) were collected from field grown-plants at Saskatoon in 2006 and freeze-dried. DNA was extracted from a 30 mg sub-sample of finely ground freeze-dried leaf tissue using DNeasy Plant Mini Kit (QIAGEN Inc, Valencia, California, USA) following the manufacturer's instructions. Both SSR and AFLP markers were used to genotype the RILs. Initially the markers were screened for polymorphism between the parental cultivars and then the polymorphic markers were scored in the RILs.

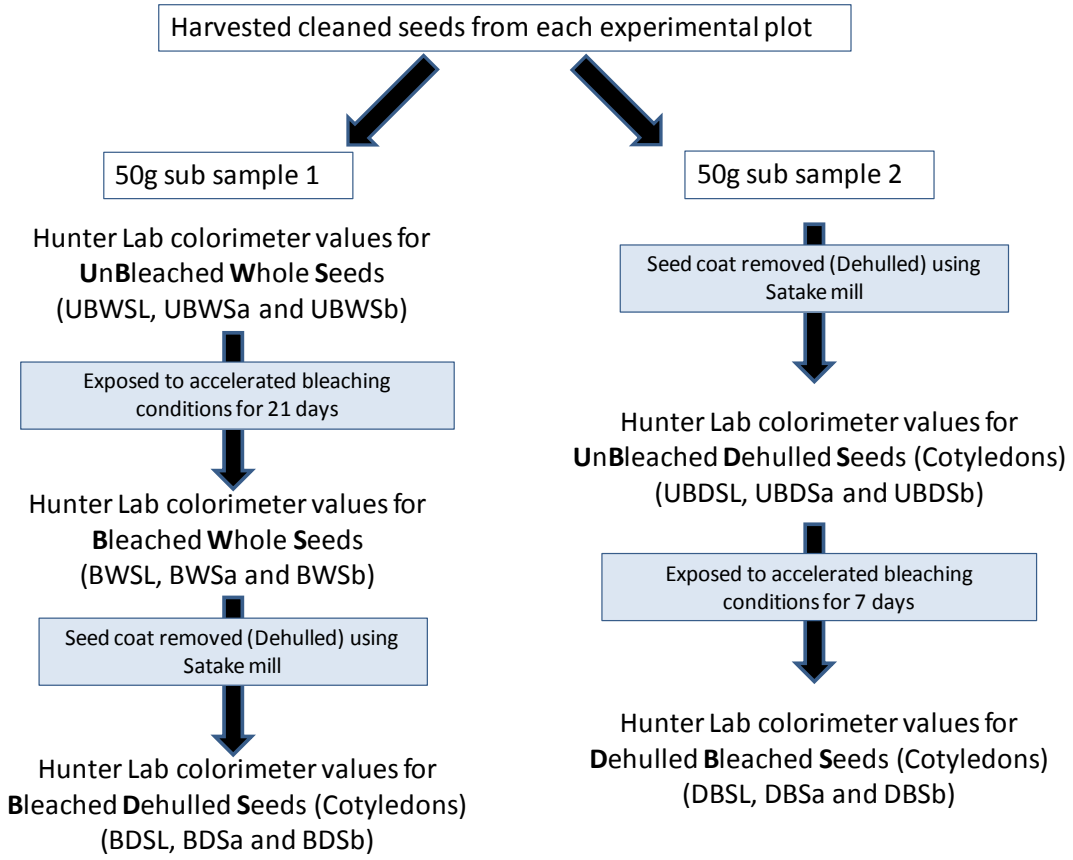

Fig. 3.1. Schematic representation of the color measurements utilized. Acronyms within parenthesis refer to the Hunter Lab colorimeter L, a, and b values measured.

3.3.3.1 SSR marker analysis

Pea microsatellite markers developed by the Agrogène Inc. consortium (Moissy-Cramayel, France) were utilized in this study. The SSR marker analysis was conducted with an ABI 377 Genetic Analyzer (Applied Biosystems Inc. Foster City, Califonia, USA). PCR for SSR analysis was conducted as described by Schuelke (2000) with slight modifications to the reaction conditions. To detect the amplified DNA fragments from SSR primer combinations, as well as to facilitate multiplexing possibilities, the forward primer sequences of each SSR primer combination were synthesized with extra M13 universal primer sequence (5'CACGACGTTGTAAAACGAC3') on the 5' end. These M13-attached forward primers were used in three-primer PCR amplifications along with the respective reverse primers and 5' fluorescently labeled (HEX, NED or FAM) M13 universal primer sequence (5'CACGACGTTGTAAAACGAC3') synthesized by Applied Biosystems Inc.

Amplification reactions were performed as reported in Somers et al. (2004) in a 20 µL reaction mixture of 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 9.0 at 25°C), 1.5 mmol/L $MgCl_2$, 0.01% Triton X-100, 0.2 mmol/L each dNTP (Invitrogen, Burlington, Ontario, Canada), 1 U of *Taq* polymerase (GenScript Corp. Piscataway, New Jersey, USA), 0.02 µmol/L M13 sequence attached forward SSR primer, 0.2 µmol/L regular reverse SSR primer, 0.18 µmol/LFAM-, HEX- or NED-labeled M13 primer and 20 ng of DNA. PCR amplification reactions were performed in a PTC-200 thermal cycler (MJ Research, Waltham, Massachusetts, USA) using the following thermal profile; 95 ^{o}C for 3 min, 4 cycles of touchdown thermal profile consisting of 4 cycles of 94 ^{o}C for 30 s, 56 ^{o}C to 50 ^{o}C (-2 ^{o}C/cycle) for 50 s, 72 ^{o}C for 50 s, and then 25 cycles of 94 ^{o}C for 30 s, 51 ^{o}C for 50 s, 72 ^{o}C for 50 s. A final extension of PCR products at 72 ^{o}C for 10 min was allowed before the final step at 8 ^{o}C. PCR products resulting from different primer combinations were then multiplexed by combining 0.3 µL of HEX-labeled PCR products, 0.3 µL of NED-labeled PCR products and 0.4 µL of FAM-labeled PCR products. These mixtures were subsequently denatured at 95 ^{o}C for 5 min after adding 0.05 µL of Rox 1000 internal standard (Applied Biosystems Inc.) and 0.5 µL of loading dye which contained 5:1 formamide and TE buffer containing Dextran Blue (Applied Biosystems Inc.).

The denatured samples (0.8 µL) were loaded and electrophoresed on 4% denaturing polyacrylamide gels in an ABI 377 Genetic Analyzer (Applied Biosystems Inc.). The data were

collected by the ABI data collection software and analyzed by the GeneScan Analysis Software of the Applied Biosystems Inc.

3.3.3.2 AFLP marker analysis

The AFLP marker analysis was performed as described by Vos et al. (1995) with minor modifications. Forty eight primer combinations made up of six *Eco*RI + 2 selective nucleotide primers (E-CA, E-AC, E-CC, E-CG, E-AG and E-CT) and eight *Mse*I +2 selective nucleotide primers (M-AA, M-AC, M-AG, M-AT, M-TA, M-TC, M-TG and M-TT) were employed in this study. The selective amplification products were separated on 6% polyacrylamide gels by electrophoresis for 4 h at 80 W in 1% TBE buffer. The gels were silver-stained after being fixed in 10% glacial acetic acid for 30 min (Bassam et al. 1991). Silver-stained gels were then scanned and scored for the markers segregating in the 90 RILs. The polymorphic bands were recorded as the two selective nucleotides of the *Eco*RI and *Mse*I primers followed by fragment length.

3.3.4 Data analysis

3.3.4.1 Phenotypic data analysis

Statistical analysis for phenotypic data was done following the PROC MIXED procedure of the SAS program (SAS Institute Inc. 1997). Genotypes were considered as fixed effects, whereas year, location, replicates and incomplete blocks were considered random effects for the estimation of means for each RIL and the parental cultivars. The parental cultivars were removed from the data sets to facilitate the estimation of variance components of the RILs without confounding them. The genotypes, locations, years, replicates and their interactions were considered random for the estimation of variance components to estimate the genetic parameters. The phenotypic variance was estimated as $\sigma^2_P = \sigma^2_G + (\sigma^2_{GY}/y) + (\sigma^2_{GL}/l) + (\sigma^2_{GLY}/ly) + (\sigma^2_e/lyr)$, where σ^2_G is the estimated genotypic variance, σ^2_{GY} is the genotype year interaction, σ^2_{GL} is the genotype location interaction, σ^2_{GYL} is the genotype, year and location interaction, y is the number of years tested, l is the number of locations, and r is the number of replicates per location. Heritability estimates for each trait were estimated as $H = \sigma^2_G / \sigma^2_P$. Phenotypic correlations among the traits were calculated using the PROC CORR procedure of the SAS program.

3.3.4.2. Linkage map construction and QTL analysis

The markers were assigned to linkage groups (LGs) at a LOD value of 6 using the Haldane map function of the Join-Map program Ver. 3.0 (Van Ooijen and Voorips 2004). The LGs identified in this study were aligned to the seven pea chromosomes based on the common markers in the previously published pea genetic map (Loridon et al. 2005). Least square means of each trait were used for QTL analysis using MapQTL5 (Van Ooijen 2004). The LOD threshold to declare significant association of the genomic regions with the trait was determined by 1000 permutations. A multiple QTL mapping method was used for the markers having the highest LOD values as cofactors to determine the true QTLs for the traits under consideration.

3.4 Results

3.4.1 Genetics of cotyledon bleaching resistance in green peas

Table 3.1 provides a partial analysis of variances with mean square values and significance for the genotypes and main environmental interaction terms that contributed to explain the phenotypic variability of the seed and cotyledon greenness at different stages of seed evaluation. Genotype had a significant ($P \leq 0.001$) effect on UBWSa, UBDSa, BWSa, BDSa and DBSa (see Fig. 3.1 for these acronyms). The genotype X year and genotype X year X location interactions were also significant for all of these measurements except DBSa. The genotype X location interaction was significant ($P \leq 0.05$) for only UBDSa. The results indicated a significant environmental influence on the seed greenness before and after bleaching. The coefficient of variation (CV) for the measured phenotypic estimates ranged from 7.3% to 25.1%. Relatively low CVs were observed for UBDSa, BDSa and DSBa compared to UBWSa and BWSa; i.e., the reliability of the seed greenness values was improved by assessing dehulled seeds compared to whole seeds. Thus, UBDSa and BDSa were utilized in estimating the genetic parameters and in QTL analysis.

Mean, standard deviations, minimum and maximum values of the Hunter Lab colorimeter "a" values of the RILs and the means for the parental cultivars over two years at two locations are given in Table 3.2. The bleaching-resistant pea cultivar CDC Striker had greater negative values for UBWSa, UBDSa, BWSa and BDSa than the bleaching-susceptible cultivar Orb.

Table 3.1. Partial analysis of variance with mean squares and significance levels for the greenness of unbleached and bleached seeds and cotyledons estimated by Hunter Lab colorimeter "a" values for the 90 RILS of the Orb X CDC Striker population grown at two locations over two years.

Effect	df	Mean squares of the seed greenness measurements				
		UBWSa	UBDSa	BWSa	BDSa	DBSa
Genotype	89	0.50^{***}	1.24^{***}	1.55^{***}	18.43^{***}	3.64^{***}
Genotype X Year	89	0.14^{**}	0.35^{***}	0.27^{**}	3.37^{**}	0.34^{NS}
Genotype X Location	89	0.07^{NS}	0.20^{*}	0.13^{NS}	2.02^{NS}	0.43^{NS}
Genotype X Year X Location	89	0.07^{**}	0.20^{**}	0.16^{*}	1.90^{**}	0.32^{NS}
CV (%)		11.60	7.30	25.10	15.00	10.80

Note: For explanation of UBWSa, UBDSa, BDSa, and DBSa, refer to Fig 3.1. NS, not significant, *= Significant at P≤0.05, ** = Significant at P≤0.01, *** : Significant at p≤0.001

Table 3.2. Mean, standard deviation (SD), minimum and maximum Hunter Lab colorimeter "a" values for the 90 RILs of the Orb X CDC Striker population and the means of the parental cultivars over two years at two locations.

	Orb X CDC Striker RIL population				Parental cultivars	
Variable	Mean	Std Dev	Minimum	Maximum	Mean	
					Orb	CDC Striker
UBWSa	-1.85	0.28	-2.31	-1.07	-1.33	-1.86
UBDSa	-4.17	0.46	-4.79	-1.89	-3.73	-4.65
BWSa	-1.32	0.49	-2.01	0.21	0.44	-1.42
BDSa	-3.27	0.83	-4.55	-0.70	-0.42	-3.96
DBSa	4.92	0.77	2.91	7.16	5.03	4.42
UBWSa- UBDSa	2.32	0.29	0.74	2.82	2.40	2.79

Note: For explanation of UBWSa, UBDSa, BWSa, BDSa, and DBSa, refer to Fig. 3.1

These results indicate that the CDC Striker seeds were greener at the time of harvesting and retained their greenness after exposure to bleaching conditions to a greater extent than Orb. Most of the Hunter Lab colorimeter estimates for the RILs were negative, indicating the samples retained green color. Extreme RILs having seeds with much more or less greenness than the parental cultivars after exposure to the bleaching conditions were observed, indicating transgressive segregation.

The DBSa values were positive for all the RILs and parental cultivars (Table 3.2). The color of these samples changed from green to faint green or yellow. This suggests that the main biological tissue protecting the cotyledons from light-mediated green color bleaching is the seed coat. An index to investigate the improvement of Hunter Lab "a" values by removing the seed coats compared with whole seeds as an indirect estimation of the seed coat translucency was calculated by taking the difference between UBWSa and UBDSa. In this index, the greater the difference, the less translucent the seed coats, and vice versa.

Table 3.3 shows the correlation coefficients among the Hunter lab colorimeter "a" values of the seed material measured at different stages of the bleaching evaluation as well as the seed coat translucency index. Highly significant ($P \leq 0.001$) correlations were observed for all of the Hunter Lab colorimeter "a" values of the whole seeds and cotyledons before and after exposure to the bleaching conditions, indicating that the greenness values of the seed materials measured at different stages were highly correlated and could be used as effective quantitative parameters to evaluate bleaching resistance in pea. The correlation coefficients between the seed coat translucency index and BDSa was negative and significant ($P \leq 0.001$). These results indicated that seed coat translucence has a significant effect on cotyledon bleaching resistance when whole seeds are exposed to light.

Table 3.4 provides the variance components and the broad sense heritability estimates for UBWSa, UBDSa, BWSa and BDSa. The observed genetic variances for these traits are in the range of 200-300% of the location, year, location X year interaction and error variances, indicating the importance of the genetic effects. The broad-sense heritability of the greenness of whole seeds and cotyledons before exposure to the accelerated bleaching conditions were 0.72 (UBWSa) and 0.69 (UBDSa), respectively. The heritability values of BWSa (0.83) and BDSa (0.82) were higher than those of UBWSa and UBDSa (Table 3.4).

Table 3.3. Correlations between the Hunter Lab colorimeter "a" values of seeds and cotyledons of the 90 RILs of the Orb X CDC Striker population over two years at two locations.

	UBWSa	UBDSa	BWSa	BDSa	DBSa	UBWSa-UBDSa
UBWSa	-	0.81***	0.89***	0.77***	0.72***	-
UBDSa		-	0.80***	0.86***	0.82***	-
BWSa			-	0.93***	0.73***	-0.44***
BDSa				-	0.76***	-0.64***
DBSa					-	-
UBWSa-UBDSa						-

Note: For explanation of UBWSa, UBDSa, BWSa, BDSa, and DBSa, refer to Fig 3.1. ***, significant at $P \leq 0.001$

Table 3.4. Estimates of variance components and heritability of the greenness measured by Hunter Lab colorimeter "a" values for whole seeds and cotyledons before and after exposure to accelerated bleaching conditions for the 90 RILs of the Orb X CDC Striker population grown at two locations over two years.

Variance component	UBWSa	UBDSa	BWSa	BDSa
σ^2_G	0.06±0.01***	0.16±0.04***	0.21±0.04***	0.61±0.11***
σ^2_{GY}	0.02±0.00**	0.07±0.02***	0.04±0.01**	0.13±0.04**
σ^2_{GL}	0.00±0.00	0.02±0.01*	0.00±0.00**	0.01±0.03^{NS}
σ^2_{GLY}	0.02±0.00***	0.03±0.01*	0.02±0.01*	0.10±0.04**
σ^2_e	0.05±0.00***	0.09±0.01***	0.11±0.01***	0.25±0.02***
σ^2_P	0.08	0.23	0.25	0.75
H^2	0.72	0.69	0.83	0.82

Note: For explanation of UBWSa, UBDSa, BWSa, and BDSa, refer to Fig 3.1. σ^2_G, genotypic variance; σ^2_{GY}, genotypic X year interaction variance; σ^2_{GL}, genotypic X location interaction variance; σ^2_{GLY}, genotypic X location X year interaction variance; σ^2_e, error variance; σ^2_P, phenotypic variance; H^2, broad-sense heritability; NS, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; ***, significant at $P \leq 0.001$.

The distribution of Hunter Lab 'a' values of the UBWSa and UBDSa are provided as box and whisker plots in Figs. 3.2 and 3.3, respectively. UBWSa and UBDSa showed continuous variation without any distinct patterns for seed greenness in both years at both locations indicating quantitative inheritance with polygenic control. The range in greenness of the whole seeds and cotyledons after harvest was greater in 2007 than in 2006 for both locations. In 2006, seeds and the cotyledons of Orb and CDC Striker had similar greenness scores, whereas in 2007, seeds of Orb were more bleached than those of CDC Striker.

Average temperature and total precipitation during the growing seasons (May to August) in 2006 and 2007 for Saskatoon and Rosthern are given in Table 3.5. In both seasons, Rosthern received more precipitation compared to Saskatoon and the 2007 growing season was wetter than 2006 in both locations. A high degree of transgressive segregation was observed for whole seed and cotyledon greenness in both years and locations. Frequency distribution of BWSa and BDSa are shown as box and whisker plots in Figs. 3.4 and 3.5, respectively. These distributions were also continuous and were skewed to the bleaching-resistant parent, CDC Striker. This indicated that the bleaching resistance after harvest was quantitatively inherited and most of the alleles contributing to the bleaching resistance were contributed by CDC Striker.

3.4.2 Molecular marker analysis and linkage map construction

Three hundred fifty pea SSR markers were screened on the DNA of the two parental cultivars. This screening identified 64 SSRs that showed polymorphism between Orb and CDC Striker. Of these SSRs, 49 yielded clear segregating loci among the RILs. Fifteen primers initially identified as polymorphic between the parents had monomorphic bands or failed to amplify with the RILs. Fifty-two loci generated by these 49 SSRs were used for the construction of the genetic linkage map. In addition, a total of 273 AFLP loci from 27 primer combinations that were polymorphic between the parental cultivars and segregating among the 90 RILs were also used for the construction of the genetic linkage map.

A genetic linkage map utilizing 224 markers (29 SSR and 195 AFLP markers) was constructed for the Orb X CDC Striker population (Fig. 3.6). One hundred one markers (23 SSR and 78 AFLP), which represents 31% of the markers, remained unlinked owing to lack of linkage with any of the formed LGs.

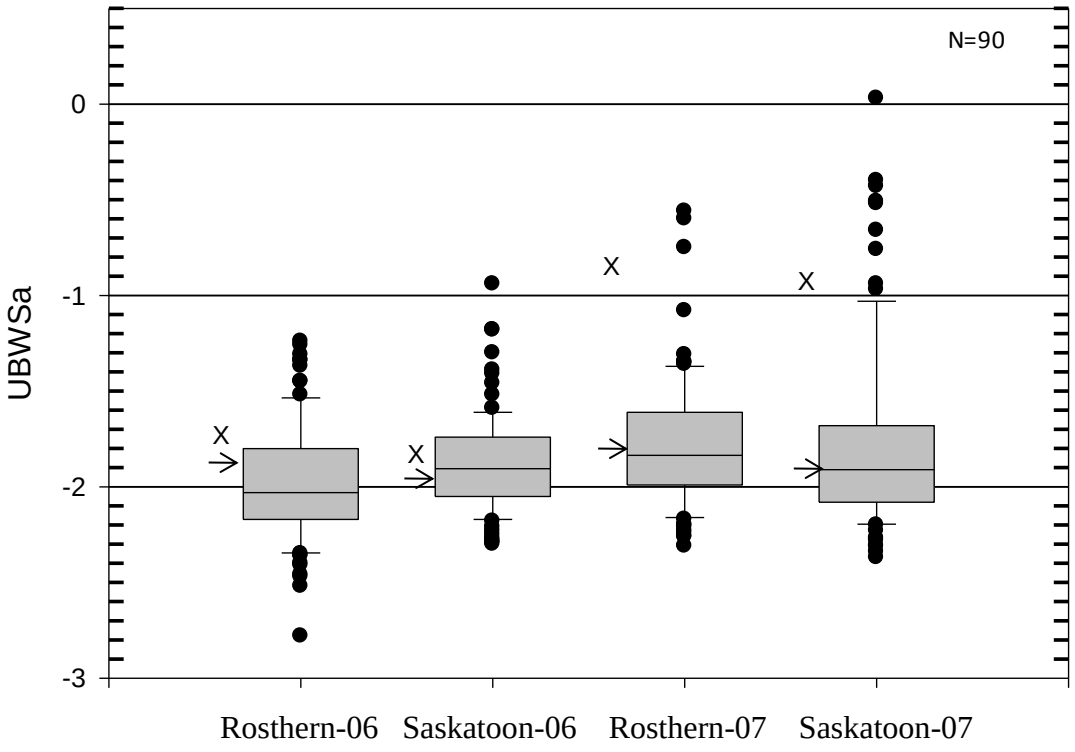

Fig. 3.2. Distribution of Hunter Lab "a" values, illustrating the greenness of whole seeds before exposure to accelerated bleaching conditions (UBWSa) for 90 RILs of the Orb X CDC Striker population at Rosthern and Saskatoon in 2006 and 2007. The boxes and the horizontal lines represent the interquartile range and the median, respectively. The whiskers attached to the boxes indicate the range of the data and the outliers indicated as dots on both sides of the whiskers. Values for Orb and CDC Striker are indicated by an X and a black arrow, respectively.

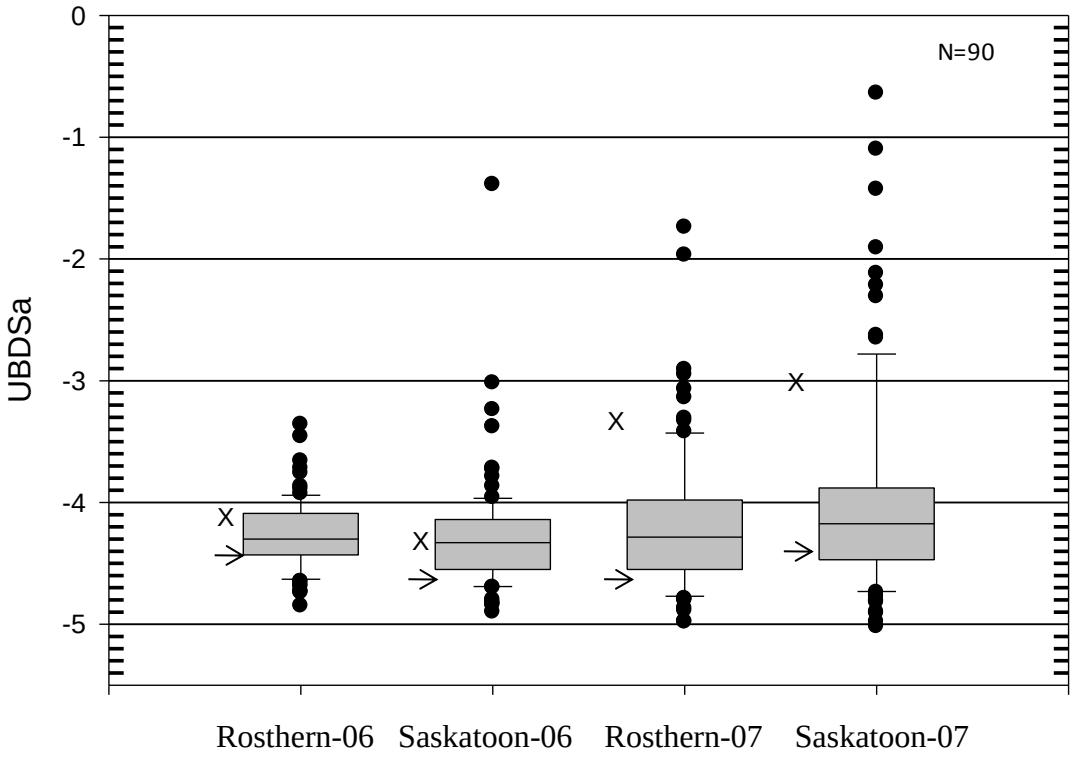

Fig. 3.3. Distribution of Hunter Lab "a" values, illustrating the greenness of cotyledons before exposure to accelerated bleaching conditions (UBDSa) for 90 RILs of the Orb X CDC Striker population at Rosthern and Saskatoon in 2006 and 2007. The boxes and the horizontal lines represent the interquartile range and the median, respectively. The whiskers attached to the boxes indicate the range of the data and the outliers indicated as dots on both sides of the whiskers. Values for Orb and CDC Striker are indicated by an X and a black arrow, respectively.

Table 3.5. Summary of growing season (May-August) mean temperature and total precipitation at Saskatoon and Rosthern, Saskatchewan, in 2006 and 2007.

Location	Nearest Environment Canada site	Soil zone	Year	Mean temperature* (°C)	Total precipitation* (mm)
Saskatoon	Saskatoon	Dark Brown	2006	16.5	210
Rosthern	Carlton	Black	2006	15.8	320
Saskatoon	Saskatoon	Dark Brown	2007	16.6	274
Rosthern	Carlton	Black	2007	15.0	346

* Based on data from the Environment Canada reporting site nearest to the trial location.

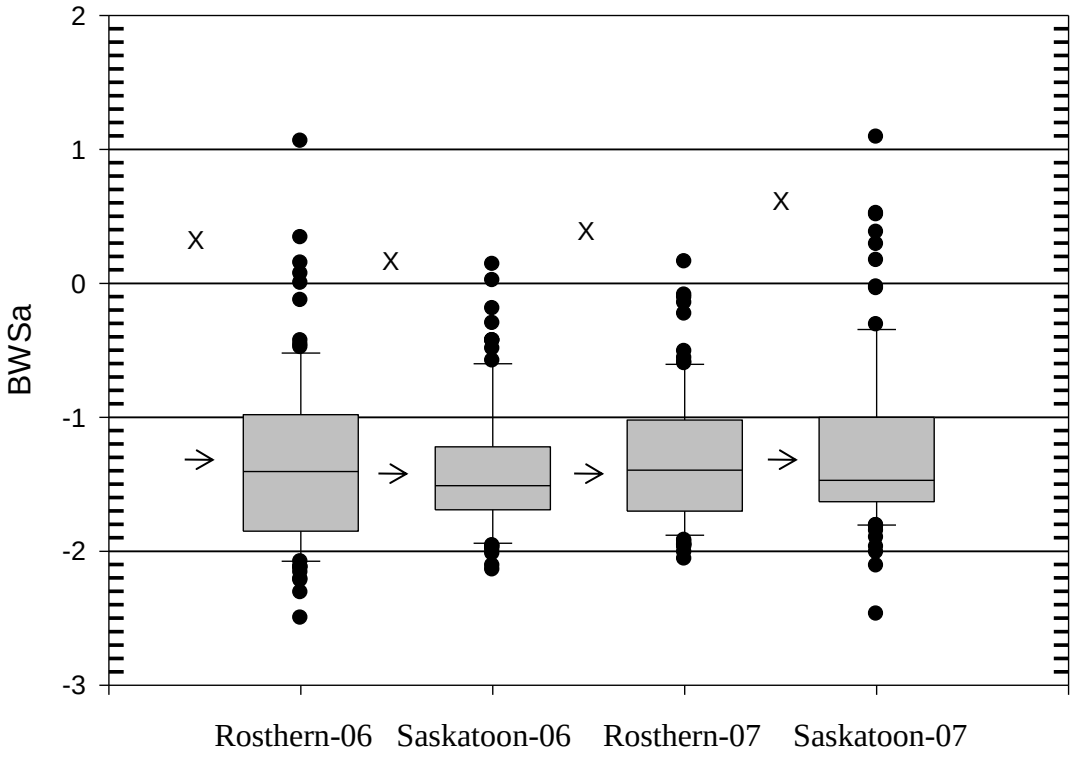

Fig. 3.4. Distribution of Hunter Lab "a" values illustrating the greenness of whole seeds after exposure to accelerated bleaching conditions (BWSa) for 90 RILs of the Orb X CDC Striker population at Rosthern and Saskatoon in 2006 and 2007. The boxes and the horizontal lines represent the interquartile range and the median, respectively. The whiskers attached to the boxes indicate the range of the data and the outliers indicated as dots on both sides of the whiskers. Values for Orb and CDC Striker are indicated by an X and a black arrow, respectively.

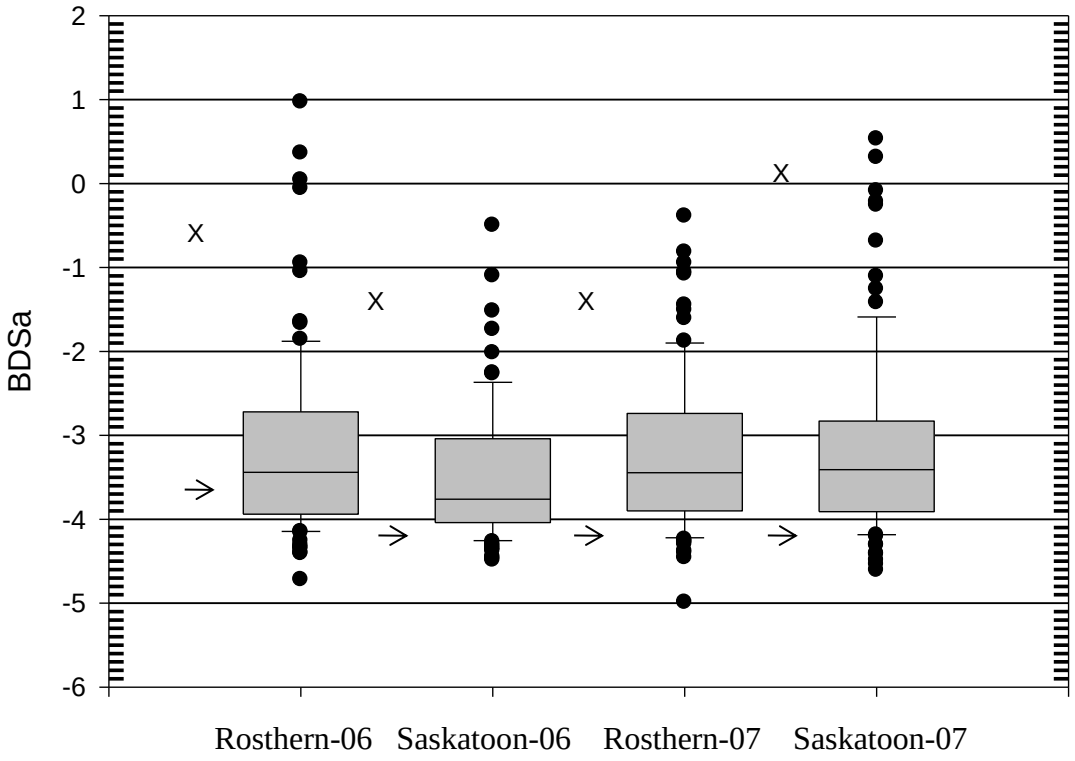

Fig. 3.5. Distribution of Hunter Lab "a" values illustrating the greenness of cotyledons after exposure to accelerated bleaching conditions (BDSa) for 90 RILs of the Orb X CDC Striker population at Rosthern and Saskatoon in 2006 and 2007. The boxes and the horizontal lines represent the interquartile range and the median, respectively. The whiskers attached to the boxes indicate the range of the data and the outliers indicated as dots on both sides of the whiskers. Values for Orb and CDC Striker are indicated by an X and a black arrow, respectively.

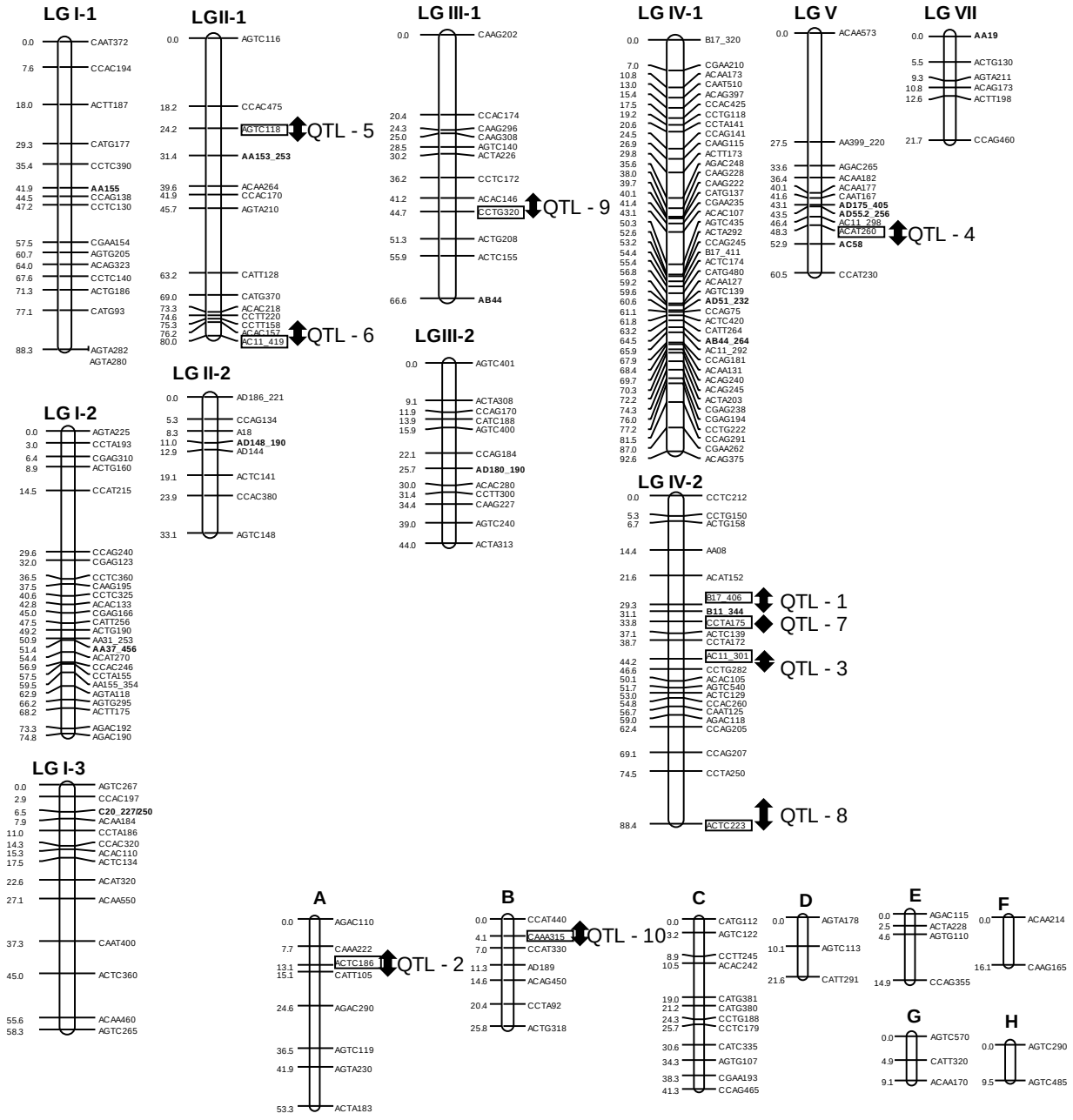

Fig. 3.6. Linkage map of the green field pea RIL population derived from a cross between Orb X CDC Striker. LG I to LG VII represent the linkage groups assigned to the seven previously described chromosomes of the pea genome using anchor markers indicated as bold text. Linkage groups from A to H are unassigned owing to lack of anchor markers. The left side of the linkage groups shows the genetic distances in centiMorgans (cM) calculated based on Haldane mapping units. Vertical arrows indicate the location of identified QTLs. The marker that mapped most closely to the detected QTL is boxed.

The map developed in this study consisted mainly of AFLP markers. Of the markers utilized in the linkage map construction, 56% of the SSR markers and 71% of the AFLP markers were assigned to LGs. AFLP markers have been utilized in genetic linkage map construction in many crops including field pea (Irzykowska et al. 2001; Tar'an et al. 2003; Tar'an et al. 2004; Gawlowska et al. 2005; Li et al. 2008). Among the 19 LGs identified in this population, 11 were aligned with 6 (LG 1, LG II, LG III, LG IV, LG V and LG VII) of the 7 LGs previously published by Loridon et al. (2005) using common SSR markers. LG 1, LG II, LG III and LG IV consisted of more than one independent segment owing to lack of marker coverage, and were indicated as LG I-1, LG I-2, LG I-3, etc. Eight LGs (A to H) could not be assigned to any of the 7 LGs of the pea genome owing to lack of anchor markers mapped to these LGs (Fig. 3.6). The total coverage of the map was 899.9 cM and the average distance between markers was 4.0 cM.

3.4.3 QTL analysis of cotyledon bleaching resistance

In order to identify the QTLs associated with bleaching resistance, greenness of the cotyledons estimated as UBDSa and BDSa was utilized. UBDSa and BDSa were identified as more precise estimates to evaluate the bleaching resistance in green peas based on the CV (Table 3.1) and the correlation analysis (Table 3.3). Putative QTL regions associated with bleaching resistance in both locations over both years are given in Fig. 3.6 and Table 3.6. A total of 10 QTLs were detected over 19 LGs (Table 3.6). A significant QTL on LG IV-2 (QTL-1) was identified for both UBDSa and BDSa across both locations and years except for the BDSa at Saskatoon in 2006. The percentage of UBDSa phenotype explained by QTL-1 ranged from 12.4% to 24.9%, and for BDSa it ranged from 5.7% to 11.9%. The molecular marker associated with the QTL-1 was SSR marker locus “B17-406” and the bleaching resistance allele was contributed by CDC Striker. The CDC Striker “B17-406” allele contribution to UBDSa ranged from -0.09 to -0.54 and for BDSa from -0.36 to -0.65 to increase the cotyledon greenness (Table 3.6).

QTL-2 associated with UBDSa was detected on LG A in 2006 at both locations. This QTL was associated with the AFLP marker locus “ACTC186” and explained 9.5% to 13.2% of the phenotypic variability at the Saskatoon and Rosthern, respectively.

Table 3.6. QTLs identified for bleaching resistance in green field pea based on the Orb X CDC Striker population in two locations over two years.

QTL region	Phenotype[a]	Year	Trail location	Linkage Group	QTL location and LOD value		Closest Marker[b]	LOD[c]	R^{2d}	Add. Effect[e]
					Location (CM)	Maximum LOD				
1	UBDSa	2006	Rosthern	IV-2	24.6-30.3	2.6^{NS}	B17-406	2.5^{NS}	10.4	-0.09 (CDC Striker)
	UBDSa	2006	Saskatoon	IV-2	24.6-30.3	2.9*	B17-406	2.8*	12.4	-0.16 (CDC Striker)
	UBDSa	2007	Rosthern	IV-2	24.6-30.3	6.0*	B17-406	6.0*	24.9	-0.29(CDC Striker)
	UBDSa	2007	Saskatoon	IV-2	24.6-30.3	4.0*	B17-406	3.4*	14.1	-0.54 (CDC Striker)
	BDSa	2006	Rosthern	IV-2	27.6-30.3	4.4*	B17-406	3.4*	11.9	-0.65 (CDC Striker)
	BDSa	2007	Rosthern	IV-2	27.6-29.3	3.6*	B17-406	3.6*	5.7	-0.36 (CDC Striker)
	BDSa	2007	Saskatoon	IV-2	25.6-30.3	4.3*	B17-406	4.2*	11.3	-0.66 (CDC Striker)
2	UBDSa	2006	Rosthern	A	12.7-15.1	3.3*	ACTC186	3.1*	13.2	0.10 (Orb)
	UBDSa	2006	Saskatoon	A	13.1-24.1	2.3*	ACTC186	2.2^{NS}	9.5	0.13 (Orb)
	BDSa	2006	Rosthern	A	9.7-15.1	3.8*	ACTC186	3.3*	11.7	0.38 (Orb)
3	BDSa	2006	Saskatoon	IV-2	42.7-46.2	3.8*	AC11-301	3.5*	12.5	-0.29 (CDC Striker)
	BDSa	2007	Rosthern	IV-2	45.2	3.5*	AC11-301	2.5^{NS}	6.7	-0.30 (CDC Striker)
4	BDSa	2006	Rosthern	V	47.4-5.30	4.2*	ACAT260	4.2*	15.2	-0.40 (Orb)
	BDSa	2007	Rosthern	V	48.2-52.3	5.0*	ACAT260	4.6*	7.5	-0.43 (Orb)
	BDSa	2007	Saskatoon	V	48.3	3.1*	ACAT260	3.1*	8.2	-0.33 (Orb)

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Cont. Table 3.6

QTL region	Phenotype[a]	Year	Trail location	Linkage Group	QTL location and LOD value		Closest Marker[b]	LOD[c]	R^{2d}	Add. Effect[e]
					Location (CM)	Maximum LOD				
5	BDSa	2007	Rosthern	II-1	22.2-25.2	3.1*	AGTC118	3.1*	4.8	0.22 (Orb)
6	BDSa	2007	Rosthern	II-1	78.2-79.9	6.0*	AC11-419	6.0*	11.8	-0.35 (Orb)
7	BDSa	2007	Saskatoon	IV-2	33.8	3.2*	CCTA175	3.2*	8.4	0.62(CDC Striker)
8	BDSa	2007	Saskatoon	IV-2	81.5-88.4	3.7*	ACTC223	3.7*	10.1	0.38 (Orb)
9	BDSa	2007	Saskatoon	III-1	41.2-50.7	3.8*	CCTG320	3.7*	9.8	-0.37 (CDC Striker)
10	BDSa	2007	Saskatoon	B	3.0-4.10	3.3*	CAAA315	3.3*	8.8	-0.35 (CDC Striker)

Note: NS, non significant QTL at $P \leq 0.05$ after 1000 permutations; *, significant QTL at $P \leq 0.05$ after 1000 permutations, [a] ,for details refer to Fig.3.1, [b] ,closest marker to the identified QTL with maximum LOD value, [c] ,LOD value of the closest marker, [d] , percentage of total variability explained by the QTL detected for the trait, [e] , additive effect for QTL detected and the responsible parent contributing to increase the value of the trait.

The “ACTC186” allele was from Orb and contributed 0.10 to 0.13 Hunter Lab “a” units to decrease the cotyledon greenness. QTL-2 was also significant for BDSa at Rosthern in 2006 and explained 11.7% of the phenotypic variability.

Eight QTLs (QTL-3 to QTL-10) were associated only with BDSa on three regions of LG IV-2, LG V, two regions of LG II-1, LG III-1 and LG B. QTL-3 was detected on LG IV-2 at Saskatoon in 2006 and at Rosthern in 2007 and the closest marker associated with this QTL was the SSR marker locus “AC11-301”. The percentage of phenotypic variation explained by this QTL was 12.5% and 6.7% and the additive effect contributed from CDC Striker was -0.29 and -0.30, respectively. QTL-4 on LG V was detected at Rosthern in 2006 and at both locations in 2006 and 2007. This QTL was associated with the AFLP marker locus “ACAT260” and explained 15.2%, 7.5% and 8.2% of the total phenotypic variability, respectively. Additive effects of this QTL ranged from -0.33 to -0.43, contributed by Orb. QTL-5 and QTL-6 located on LG II-1 were detected at Rosthern in 2007. QTL-5 was associated with the AFLP marker locus “AGTC118” and contributed 4.8% of the phenotypic variability. The allelic contribution for this QTL was from Orb to reduce the cotyledon greenness by 0.22. In contrast, QTL-6, associated with the SSR marker locus “AC11-419”, explained 11.8% of the phenotypic variability and the allele was contributed by Orb to increase the cotyledon greenness by -0.35. QTL-7 and QTL-8 were located on LG IV-2 and were detected only at Saskatoon in 2007; they explained 8.4% and 10.1% of the phenotypic variability, respectively. The allelic contribution for these two QTLs were 0.62 (CCTA175) and 0.38 (ACTC223) from CDC Striker and Orb, respectively, to reduce the cotyledon greenness. Finally, QTL-9 on LG III-1 and QTL-10 on LG B, accounting for 9.8% and 8.8% of the phenotypic variability were associated with AFLP markers loci “CCTG320” and “CAAA315”, respectively. Alleles of both of these QTLs were contributed by CDC Striker and increased cotyledon greenness by -0.37 and -0.37, respectively.

Total phenotypic variation explained by the detected QTLs for both locations over both years is given in Table 3.7. The proportion of phenotypic variability explained by the detected QTLs associated with UBDSa ranged from 13.2% (2006, Rosthern) to 24.9 (2007, Rosthern), whereas the proportion of phenotypic variability explained by the detected QTLs controlling BDSa ranged from 12.5% (2007, Saskatoon) to 56.6 % (2007, Saskatoon).

Table 3.7. Amount of phenotypic variability explained by the detected QTLs for two locations over two years for the 90 RILs of the Orb X CDC Striker population.

Phenotype[a]	Year	Location	Total r^{2} [b]
UBDSa	2006	Rosthern	13.2
	2006	Saskatoon	21.0
	2007	Rosthern	24.9
	2007	Saskatoon	14.1
BDSa	2006	Rosthern	38.8
	2006	Saskatoon	12.5
	2007	Rosthern	36.5
	2007	Saskatoon	56.6

Note: [a] , Refer to Fig. 3.1 for details, [b] ,The amount of phenotypic variation explained by the detected QTLs.

3.5 Discussion

Green cotyledon bleaching in field pea has been demonstrated to result from a decrease in chlorophyll pigment content of the cotyledons during seed maturation or post-harvest storage (Holden 1965; Riehle and Muelbauer 1975; McCallum et al. 1997). Cheng et al. (2004) observed a logarithmic relationship to the change in chlorophyll content of green pea cotyledons and the Hunter Lab colorimeter "a" values. In this study, Hunter Lab colorimeter "a" values were used to estimate the genetic parameters with respect to bleaching resistance. The overall greater bleaching observed in 2007 compared with 2006 may be explained by the differences in growing season precipitation. In 2007, both locations received more precipitation than in 2006. Wet weather conditions or irrigation during the seed maturation period are favorable for green pea bleaching (Riehle and Muelbauer, 1975; Gubbels and Ali-Khan, 1990). A strong correlation between resistance to soaking and color retention ability was demonstrated in green peas by Gubbels and Ali-Khan (1990).

Previous studies related to green pea bleaching did not address the effect of a genotype X environmental interaction during the seed maturation period on bleaching resistance (Maguire et al. 1973; Dribnenki 1979; Mepsted et al. 1996; McCallum et al. 1997; Cheng et al. 2004). The observed continuous distribution of the phenotypic measurements indicated that bleaching resistance in green peas is quantitatively inherited under polygenic control. The population of green pea RILs showed transgressive segregation for bleaching resistance. This could be explained by the large effect of genotype compared with the effects of location, year and their interaction with genotypes (Table 3.4). The occurrence of transgressive segregation for bleaching resistance in this population could be due to the additive effects of the alleles contributed by the parental cultivars to the RILs (Snoad and Arthur 1973; deVicente and Tanksley 1993; Orf et al. 1999). It could also be due to the presence of several QTLs controlling bleaching resistance or epistatic interactions between them (Salas et al. 2006). Therefore, molecular markers linked with QTLs controlling bleaching resistance could be used in pea breeding programs to identify parental lines that may not be phenotypically superior but which carry QTLs of interest.

Riehle and Muelbauer (1975) described the constraints faced by pea breeders in identifying segregants with bleaching resistance, since the expression of this resistance is highly influenced by environmental factors during the seed maturation period. In this research, the

heritability estimates of the UBWSa and UBDSa indicated that the phenotypic variation observed for the greenness of the seeds and cotyledons were influenced by environmental factors. However, the contribution of genetic factors to the total variability in bleaching resistance in green pea was much more important than the contribution of location and year or their interactions. The heritability estimates based on the BWSa and BDSa were improved compared with those based on UBWSa and UBDSa, indicating that the uniform accelerated bleaching treatment reduced the environmental variability and thereby highlighted the true genetic variability. The remaining environmental variability related to bleaching resistance could be explained by the genotype X environment interaction, which occurred during the seed developmental stages and influenced the biochemical and physical nature of the seeds with respect to bleaching resistance. The high heritability estimates of the BWSa and BDSa indicated that the genetic contribution to the total variability is high, suggesting field pea bleaching resistance is conditioned by a few genes with major effects.

The improved heritability estimates of the greenness of the bleached whole seeds and cotyledons compared with the unbleached whole seeds and cotyledons highlighted the effectiveness of using an accelerated bleaching procedure in early generation selection in field pea breeding. This also suggested the option of using the greenness of bleached whole seeds instead of cotyledons to select bleaching-resistant phenotypes in breeding programs, to avoid the extra effort of removing the seed coats after exposure to accelerated bleaching conditions.

The observed greenness of the cotyledons exposed to accelerated bleaching conditions without seed coats was highly correlated with the initial greenness of the cotyledons at harvest. Furthermore, the rate of loss of greenness was much greater in the cotyledons exposed to accelerated bleaching conditions than in the whole seeds. This indicated that bleaching resistance is influenced by the initial chlorophyll content of the cotyledons, and that seed coat characteristics play an important role in protecting the cotyledon chlorophyll from bleaching. The genetic factors that influence the structural and biochemical differences of the seed coats and cotyledons during seed development and maturation play a major role in bleaching resistance in field pea. Significantly different pigment accumulation and degradation rates and chlorophyll a:b ratio between green cotyledon bleaching resistant and susceptible field pea cultivars were reported in previous studies (McCallum et al. 1997). Dribnenki (1979) reported that water imbibition rate and seed coat color were two of the three main mechanisms involved in green pea

bleaching resistance. The content of phenolic compounds in developing pea seed coats and their level of oxidation during seed desiccation were significantly correlated with seed coat permeability to water (Marbach and Mayer 1974). These authors also suggested that the ecological conditions during seed maturation, which influence access of oxygen to the drying seeds, affect the oxygen-dependant phenol oxidation by catechol oxidase and pea seed coat permeability to water. The degree of seed coat translucence due to seed coat pigments or structure could influence the color of the cotyledons (McCallum et al. 1997). Heavily pigmented and less translucent seed coats have the ability to protect the underlying cotyledons from bleaching by reducing the light intensity on the cotyledon tissues (Dribnenki 1979). The observed significant negative correlation coefficients between the seed coat translucence index and BDSa also suggested that seed coat translucence has a significant effect on cotyledon bleaching resistance when whole seeds were exposed to light.

The total coverage of the linkage map generated in this study was 899.9 cM, which is considerably smaller than the maps previously reported for pea, which range from 1104 cM to 2416 cM (McCallum et al. 1997; Laucou et al. 1998; Irzykowska et al. 2001; von Stackelberg et al. 2003; Tar'an et al. 2003, 2004; Loridon et al. 2005; Aubert et al. 2006). However, the coverage of the linkage map described in this study is similar to that of the linkage map described by Timmerman-Vaughan et al. (2004) using 148 individuals of an F_3 population derived from a cross between A26 and Rover utilizing RAPD, RFLP, AFLP and STS markers, which covers 930 cM with the average distance between markers of 10.8 cM. The average distance between markers in the genetic linkage map described in this study is 4.0 cM and this could be a result of more markers being mapped on the current map than on the Timmerman-Vaughan et al. (2004) map, which was composed of 99 marker loci. Laucou et al. (1998) suggested that the differences in linkage intensity in different crosses could result in differences in linkage map coverage. The relatively low linkage map coverage and frequency of SSR marker polymorphism observed for this population may suggest a close genetic relationship of the parental lines. The genetic constitution of different mapping populations, mapping strategies, the number of mapped loci, and the choice of mapping software were described as significant factors contributing to differences in barley genetic maps (Li et al. 2008). In the genetic linkage map presented in this report, several linkage groups were split into several fragments. Lack of

sufficient neighboring polymorphic markers or integrity of these linkage groups could be possible explanations (Kosterin 1993; Kosterin and Rozov 1993; Weeden et al. 1993).

This study identified several QTLs with additive effects associated with green cotyledon bleaching resistance in field pea. The QTLs detected for UBDSa accounted for 24.9% of the variability at Rosthern in 2007, while the QTLs detected for BDSa accounted for 56.6% of the variability at Saskatoon in 2007. The broad sense heritability for UBDSa and BDSa was estimated as 0.69 and 0.82, respectively. This indicates the high potential of utilizing markers for the QTLs associated with BDSa in marker-assisted breeding. Furthermore, 8 of the 10 QTLs detected were associated with BDSa, indicating the importance of utilizing an effective method of artificial bleaching to evaluate and select transgressive segregants from breeding populations.

The inconsistency of detecting QTLs associated with BDSa for all the tested environments indicated that not only the environmental conditions during the bleaching period but also the seed developmental stages within the pods could have a significant effect on the seeds' ability to retain green color during storage. We came to this conclusion because the environmental conditions were kept constant during the course of this study. A significantly different rate of accumulation and breakdown of photosynthetic pigments were reported in bleaching-resistant cultivar OSU442-15 compared with the bleaching-susceptible cultivar Promo (McCallum et al. 1997). These authors also suggested that the genes controlling seed color have significant effects on pigment concentration in a quantitative mode of inheritance and a major QTL on LG V was identified controlling photosynthetic pigment composition. Similarly, a significant QTL associated with bleaching resistance was identified on LG V in three of the four different environments tested in this study. McCallum et al. (1997) reported that locus *pa*, responsible for bright green foliage, pod and seed color (Lamprecht 1957), mapped to the same region of LG V.

Four QTLs associated with bleaching resistance in field pea were identified on LG IV-2, and of these, QTL-1 seems most important. QTL-1 was detected using the phenotypic values of UBDSa from both locations over both years (although 2006 at Rosthern was not significant), as well as BDSa at both locations over both years, except 2006 at Saskatoon. The observed variability of UBDSa may be due to differential enzymatic degradation of chlorophyll from the cotyledons during the seed maturation and desiccation period. McCallum et al. (1997) identified

a major QTL on LG IV and suggested a close association with *Lox-10* (Arens et al. 1973; Ellis et al. 1993), which is responsible for the co-oxidation of chlorophylls and carotenoids.

Two other QTLs identified in this study on LG IV-2 (QTL-3 and QTL-7), which mapped within close proximity to QTL-1 could be the other two loci described by McCallum et al. (1997) and linked with the genes *vim* and *olv*, which give dark green phenotypes with the recessive allele of *pa* (Lamprecht 1957) and greenish gray seed coats (Blixt 1962), respectively. QTL-8, found on the proximal end of LG IV-2, which was responsible for controlling 10.1% of the total phenotypic variation, could be a specific QTL for the Orb X CDC Striker population. McCallum et al. (1997) could not clearly identify LG III and LG IV owing to co-segregation of markers utilized to develop the linkage map. In contrast, the linkage map presented here clearly recognized LG III and LG IV and also identified QTL-9 on LG III contributes to bleaching resistance. The results of this study demonstrated that the genes on LG III, IV and LG V made significant contributions to controlling bleaching resistance in green pea.

In addition to the previously identified QTLs associated with bleaching resistance, four more QTLs with significant contribution to bleaching resistance have been identified on LG II-1 (QTL-5 and QTL-6), LG A (QTL-2) and LG B (QTL-10). Due to lack of mapped anchor markers, LG A and LG B could not be designated to any of the seven linkage groups in pea. This highlights the need to saturate the current linkage map with more molecular markers to get reasonable coverage of the whole genome of field pea.

Our results clearly demonstrated that additive genetic contributions were from both parents. Bleaching resistance alleles were mainly contributed from CDC Striker, while Orb contributed alleles for bleaching susceptibility in most of the instances. Identification of QTLs with both positive and negative additive genetic effects could explain the occurrence of transgressive segregants having extreme phenotypes in both directions. This indicated the possibility of improving pea cultivars by pyramiding these QTLs with positive additive genetic effects. This also highlighted the possibility of utilizing molecular markers associated with these QTLs in marker-assisted breeding to improve the efficiency of breeding programs, by identifying individuals with the correct loci combination and by reducing the phenotyping cost and time. However, further validation of these markers linked with the identified QTLs using different genetic backgrounds is recommended.

CHAPTER 4

4. Genetic control and identification of QTLs associated with visual quality traits of field pea (*Pisum sativum* L.)

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4.1 Abstract

Visual quality of field pea *(Pisum sativum L.)* is one of the most important determinants of the market value of the harvested crop. Seed coat color, seed shape and seed dimpling are the major components of visual seed quality of field pea and are considered as important breeding objectives. The objectives of this research were to study the genetics and to identify quantitative trait loci (QTLs) associated with seed coat color, seed shape and seed dimpling of green and yellow field pea. Two recombinant inbred line populations (RILs) consisting of 120 and 90 lines of F_5 derived F7 (F5:7) yellow pea (*P. sativum* 'Alfetta' X *P. sativum* 'CDC Bronco') and green pea (*P. sativum* 'Orb' X *P. sativum* 'CDC Striker'), respectively, were evaluated for two years at two locations in Saskatchewan, Canada. Quantitative inheritance with polygenic control and transgressive segregation were observed for all visual quality traits studied. All 90 RILs of the green pea population and 92 selected RILs from the yellow pea population were screened using AFLP and SSR markers and two linkage maps were developed. Nine QTLs controlling yellow seed lightness, 3 for yellow seed greenness, 15 for seed shape and 9 for seed dimpling were detected. Among them, five QTLs located on LG II, LG IV and LG VII were consistent in at

least two environments. The QTLs and their associated markers will be useful tools to assist pea breeding programs attempting to pyramid positive alleles for the traits.

Key words: Field pea, Visual quality, Seed color, seed shape, Seed dimpling, Amplified Fragment Length Polymorphisms, Simple Sequence Repeats, QTL mapping

4.2 Introduction

Visual appearance of harvested seeds plays an important role in determining the market value of many agricultural crops. Among visual quality traits, seed shape, color and seed coat texture are usually the most important (Salas et al. 2006; Yan et al. 2009; Bhattacharyya et al. 1993). For both green and yellow cotyledon field pea (*P. sativum* L.), seed color is a critical determinant of grade and hence the market value (Canadian Grain Commission 2008). To qualify for the highest market grade (Canada No.1), green pea seeds should have a natural green color with less than 2% bleached seeds, whereas yellow pea should have natural yellow color with less than 1% other seed colors, such as green or orange. Other than seed color, seed shape (i.e., round, as opposed to blocky or angular shape) and seed coat texture (i.e., smooth, as opposed to dimpled, or "golf-ball") are often considered by pulse traders beyond the Canadian Grain Commission grading standards. In addition, seed size and uniformity also plays an important role in field pea trading.

Development of pea varieties with improved seed quality is one of the most important objectives in pea genetic improvement programs (McPhee 2007). However, genetic improvement of these quality traits is challenging to the quantitative mode of inheritance and significant influence of environmental factors on the expression of the desired phenotype under field growing conditions. Therefore, understanding the genetics, identification of quantitative trait loci (QTLs) and development of molecular markers for the selection of visual quality traits would be beneficial. Significant progress in constructing genetic linkage maps and locating 72 QTLs (McPhee 2007) associated with several economically important traits in field pea, including lodging resistance, plant height, *Mycosphaerella* blight resistance, seed weight, green seed color, grain yield, seed protein content, maturity, resistance to *Aphanomyces euteiches*, resistance to *Orobanche crenata* have been reported to date (Timmerman-Vaughan et al. 1996;

McCallum et al. 1997; Pilet-Nayel et al. 2002; Tar'an et al. 2003; Prioul et al. 2004; Tar'an et al. 2004; Timmerman-Vaughan et al. 2004; Valderrama et al. 2004). However, neither QTLs nor molecular markers have been reported for pea visual quality traits such as greenness of mature yellow cotyledon seeds, seed shape and seed dimpling.

Domoney et al. (2006) reviewed the genetics and genomic basis of legume flower and seed development and highlighted the importance of understanding the genetic control of seed and flower development to manipulate seed yield and quality. Mechanical and textural characteristics of the testa are the major determinants of the appearance of the seed surface, in terms of smoothness compared to dimpled. Pectic polysaccharide domains in cells and tissues of the testa play an important role in maintaining the mechanical properties of developing pea seeds, especially at later stages of development (McCartney and Knox 2002). Involvement of a single gene (*mifo*) controlling the dimpling trait of pea seeds was reported by Lamprecht (1962); however, no environmental effects were assessed and no user-friendly markers were developed.

The objectives of the current research were to study the genetic and environmental effects on three visual quality traits that determine the market value of field pea (i.e., greenness in yellow pea, seed shape and seed dimpling in both yellow and green peas) and to identify QTLs associated with these traits.

4.3 Materials and Methods

4.3.1 Plant materials

Two recombinant inbred (RILs) populations of F_5 derived F_7 ($F_{5:7}$) generation were developed using single seed decent method. The green cotyledon pea RIL population consisted of 90 individuals originating from a single F_1 plant of a cross between *P. sativum* 'Orb' and *P. sativum* 'CDC Striker' (Ubayasena et al. 2010). The yellow cotyledon pea RIL population consisted of 120 individuals originating from a single F_1 plant of a cross between *P. sativum* 'Alfetta' and *P. sativum* 'CDC Bronco'. Orb is a green pea cultivar developed by Sharpes, UK with somewhat blocky and dimpled seeds. CDC Striker is a green pea cultivar developed by Crop Development Centre (CDC), University of Saskatchewan, Canada, with round seed shape and smooth seed coat surface (Warkentin et al. 2004). Alfetta is a yellow pea cultivar developed by Cebeco Zaden, the Netherlands, that tends to have moderate greenness in mature seeds and

somewhat blocky seed shape. CDC Bronco is a yellow pea cultivar developed by CDC, with round seed shape, relatively smooth seed coat surface and bright yellow color (Warkentin et al. 2005).

4.3.2 Evaluation of RILs for visual quality traits

4.3.2.1 Field evaluation

The two RIL populations along with their respective parents were evaluated for seed quality traits based on harvested seeds from field experiments conducted in 2006 and 2007 in two environments in western Canada (i.e., Saskatoon, Saskatchewan, located in the Dark Brown soil zone, and Rosthern, Saskatchewan, located in the Black soil zone). Field experiments were laid out using the first two replicates of a 10 X 10 and 12 X 12 simple lattice design with a plot size of 1 m^2 (micro-plots) for Orb X CDC Striker and Alfetta X CDC Bronco populations, respectively. Parental lines of the RILs were used to complete the simple lattice design in each experiment. At 95% pod maturity, individual microplots were hand harvested and threshed, and two representative subsamples (150 g subsample to determined the seed shape and 50 g subsample to determine the seed color-related traits and seed dimpling estimates) were drawn for seed quality evaluations.

4.3.2.2 Assessment of quality traits

Seed-color- related traits were evaluated by screening a 50 g subsample using a Hunter Lab colorimeter (Hunter Associates Lab Inc., Reston, Virginia). Values produced by the Hunter Lab colorimeter correspond to the ganglion cells of the human eye's sense of lightness (*L*), redness-greenness (*a*), and yellowness-blueness (*b*) (Marcus, 1998). Each sample was scanned three times and the average values of *L*, *a*, and *b* were used in the data analysis. Seed color of yellow pea was also visually inspected and assessed using scores from 1 to 5 (1 where 100% of seeds had bright yellow appearance, 2 where more than 90% of the seeds had bright yellow appearance, 3 where more than 70% of the seeds were slightly greenish, 4 where more than 70% of the seeds were moderately greenish, 5 where more than 95% of the seeds were greenish) for validation of Hunter Lab readings.

Seed shape of both populations was determined by estimating the percentage of round seeds using the 150 g subsample. Percentage of round and blocky seeds were estimated by separating the subsample using spherical-nonspherical seed sorter (Agriculex SNS-1; Agriculex Inc., Guelph, Ontario). The average of three readings for each sample was used in the data analysis.

Seed dimpling was assessed visually on a scale of 1 to 5 (1 where more than 95% of seeds had a smooth surface, 2 where approximately 10% of the seeds were lightly dimpled, 3 where approximately 30% of the seeds were lightly to moderately dimpled, 4 where approximately 50% of the seeds were moderately to severely dimpled, and 5 where more than 95% of the seeds were severely dimpled) using a 50 g seed subsample. Both the presence of dimples as well as the intensity of the dimpling, was considered when assigning visual scores.

Phenotypic data were analyzed using the PROC MIXED procedure of SAS (SAS Institute, Inc., 1997). The effect of genotype was considered as fixed, whereas year, location, replicate and incomplete block were considered random for the estimation of means for each RIL and the parental cultivars. The variance components of the genotype and the main interactions with the genotype (genotype and year, genotype and location, genotype, year, and location) were estimated by considering all the factors of the PROC MIXED model as random factors. Heritability estimates for each trait were estimated as $H = \sigma^2_G / \sigma^2_P$, where σ^2_G and σ^2_P were genotypic and phenotypic variance, respectively. The phenotypic variances were calculated by the equation, $\sigma^2_P = \sigma^2_G + (\sigma^2_{GY}/y) + (\sigma^2_{GL}/l) + (\sigma^2_{GLY}/ly) + (\sigma^2_e/lyr)$, where σ^2_G is the estimated genotypic variance, σ^2_{GY} is the genotype X year interaction variance, σ^2_{GL} is the genotype X location interaction variance, σ^2_{GYL} is the genotype X year X location interaction variance, σ^2_e is the error variance, y is the number of years tested, l is the number of locations, and r is the number of replicates per each location. The SAS procedure PROC CORR was used to conduct phenotypic correlations.

4.3.3 Linkage mapping and QTL analysis

The genetic linkage map described in Ubayasena et al. (2010) for the Orb X CDC Striker population was utilized in this study to map the QTLs associated with seed shape and dimpling in green cotyledon peas. Ninety-two of the 120 RILs from the Alfetta X CDC Bronco population evaluated in the field experiments were randomly selected and utilized in the molecular marker

analysis for the construction of a genetic linkage map. This was done mainly to accommodate the 96-well PCR format. Isolation of DNA, SSR and AFLP analyses, genetic linkage map construction and QTL analysis were conducted as described by Ubayasena et.al. (2010). In brief, pea SSR primers developed by Agrogene Inc. (Moissy-Cramayel, France) were used to genotype the RIL populations. AFLP analysis was performed using 29 primer combinations made up by six *Eco*RI + 2 selective nucleotide primers (E-CA, E-AC, E-CC, E-CG, E-AG and E-CT) and eight *Mse*I +2 selective nucleotide primers (M-AA, M-AC, M-AG, M-AT, M-TA, M-TC, M-TG and M-TT) using the methods described in Tar'an et al. (2003). AFLP marker bands that were polymorphic between parental lines and segregated within each RIL population were scored and recorded as the two selective nucleotides of the *Eco*RI and *Mse*I primers followed by fragment length for the linkage map construction. Construction of the genetic map and QTL mapping utilizing the least square means of each trait were performed using JoinMAP® 3.0 (Van Ooijen and Voorips 2004) and MapQTL® 4.0 (Van Ooijen 2004) computer software as described by Ubayasena et al. (2010).

4.4 Results

4.4.1 Genetic analysis of visual quality traits

Table 4.1 and 4.2 illustrate partial analyses of variance indicating the mean square values for genotype and major interaction terms that contributed to the total phenotypic variability for seed shape, seed dimpling and seed color in the Orb X CDC Striker and Alfetta X CDC Bronco populations at two locations over two years, respectively. The genotypic differences for seed shape measured by the percentage of round seeds were significant ($P \leq 0.0001$) for both RIL populations. There was a significant ($P \leq 0.05$) genotype X year interaction for the Alfetta X CDC Bronco population (Table 4.2), whereas for the Orb X CDC Striker population it was not significant (Table 4.1). For both populations, the genotype X location interaction was not significant ($P \leq 0.05$). However, the genotype X year X location interaction was significant for both populations ($P \leq 0.05$). The genotype, genotype X year, and genotype X year X location interactions were significant for Hunter Lab colorimeter *L* (lightness) and *a* (greenness) values (Table 4.2). Genotype X location interaction was also significant for greenness.

Table 4.1. Partial analysis of variance with mean squares and significance levels for seed shape and seed dimpling for the 90 RILs of the Orb X CDC Striker population grown at two locations over two years.

Effects	df	Mean squares	
		Seed shape[a]	Seed Dimpling[b]
Genotype	89	355.3***	1.9***
Genotype X Year	89	39.4NS	0.7NS
Genotype X Location	89	33.4NS	0.7NS
Genotype X Year X Location	89	42.1***	0.5*
CV (%)		7.2	27.2

Note: NS, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; ***, significant at $P \leq 0.001$.; CV, coefficient of variation.

[a]Percentage of round seeds.

[b]Visual scale for seed dimpling where 1 represents more than 95% of seeds having a smooth surface to 5 representing more than 95% of the seeds being severely dimpled.

Table 4.2. Partial analysis of variance with mean squares and significance levels for seed shape, lightness, greenness, and seed dimpling for the 120 RILs of the Alfetta X CDC Bronco population grown at two locations over two years.

Effects	df	Mean squares of seed visual quality traits				
		Seed shape[a]	Seed color			Dimpling [e]
			Lightness[b]	Greenness[c]	Visual greenness[d]	
Genotype	119	261.7***	5.5***	0.9***	2.6***	4.8***
Genotype X Year	119	31.7*	1.6*	0.3***	0.7NS	0.6NS
Genotype X Location	119	23.2NS	1.2NS	0.2***	0.5NS	0.5NS
Genotype X Year X Location	119	21.6**	1.3*	0.2***	0.6NS	0.8***
CV (%)		5.4	1.7	5.9	28.3	23.7

Note: NS, not significant; *, significant at $P \leq 0.05$; **, significant at $P \leq 0.01$; ***, significant at $P \leq 0.001$.; CV, coefficient of variation.

[a]Percentage of round seeds.

[b]Hunter Lab *L* value where 0 is black and 100 is white.

[c]Hunter Lab *a* value where negative values are increasingly green and positive values are increasingly red

[d]Visual scale for seed greenness where 1 represents 100% of seeds had bright yellow appearance to 5 representing more than 95% of the seeds having greenish color.

[e]Visual scale for dimpling where 1 represents more than 95% of seeds having a smooth surface to 5 representing more than 95% of the seeds being severely dimpled.

In terms of the visual assessment of greenness, genotypes differed significantly, whereas the interaction terms were not significant. Analysis of variance of seed dimpling indicated that the variation owing to genotype and genotype X location X year were significant for both populations (Table 4.1 and 4.2). These results indicated significant environmental and genotype X environmental interaction on the expression of seed visual quality traits in field pea.

Table 4.3 summarizes the mean, standard deviation, and range for the visual quality traits for both RIL populations along with the mean value of the respective parental cultivars over two years at two locations. The percentage of round seeds for Orb (57.7%) and CDC Striker (75.5%) were significantly different ($P \leq 0.05$), whereas for Alfetta and CDC Bronco, the seed shape was not significantly different ($P \leq 0.05$) and round seed percentages were 76.3% and 75.7%, respectively. However, the two RIL populations derived from these parental cultivars displayed a substantial range for seed shape (Orb X CDC Striker population: 51.6%-87.2% round seeds and Alfetta X CDC Bronco population: 57.7%-85.9% round seeds). The RIL population derived from Alfetta X CDC Bronco ranged from 52.5-57.9 for lightness, 4.6-6.7 for greenness and 1.0 to 4.3 for visual greenness. Both lightness and greenness values were significantly different among parents ($P \leq 0.001$), whereas visual greenness values were not. Seed dimpling of the 'Alfetta' X 'CDC Bronco' population ranged from 1.2 to 4.8, whereas the 'Orb' X 'CDC Striker' population ranged from 1.3 to 3.9. For all visual quality traits investigated in this research, several RILs were identified which were outside the range of the respective parents. The frequency distributions of all six phenotypic estimates evaluated are shown in Fig 4.1. All the visual quality traits in both populations showed continuous distribution suggesting polygenic control and quantitative inheritance. As expected, seed greenness (lower values indicate greenness) was negatively correlated ($P \leq 0.001$) with visual greenness (higher values indicate greenness) in the yellow pea RIL population (Table 4.4). A negative correlation ($P \leq 0.001$) was also observed between seed shape and dimpling (i.e., seeds that were rounder tended to be less dimpled).

The broad-sense heritability estimates for seed shape were 0.90 and 0.87 for the Orb X CDC Striker and Alfetta X CDC Bronco populations, respectively, indicating the majority of the phenotypic variability was due to genetic contributions.

Table 4.3. Mean, standard deviation, minimum and maximum values for several visual quality traits of field pea RILs and the means of the respective parental cultivars over two years at two locations.

RIL population	Quality Trait	RIL population			Parental means			
		Mean	Std Dev	Range	Orb	CDC Striker	Alfetta	CDC Bronco
Orb X CDC Striker	Seed Shape	72.4	7.2	51.6 - 87.2	57.7	75.5	-	-
	Seed Dimpling	2.4	0.6	1.3 - 3.9	2.4	1.5	-	-
Alfetta X CDC Bronco	Seed Shape	72.4	6.1	57.7 - 85.9	-	-	76.7	75.7
	Seed Dimpling	2.8	0.8	1.2 - 4.8	-	-	3.8	1.9
	Lightness	56.0	0.9	52.5 - 57.9	-	-	56.7	55.1
	Greenness	5.7	0.4	4.6 - 6.7	-	-	6.3	5.9
	Visual greenness	2.6	0.6	1.0 - 4.3	-	-	2.1	2.2

Note: For explanation of seed shape, seed dimpling, lightness, greenness, and visual greenness, refer to Table 4.2.

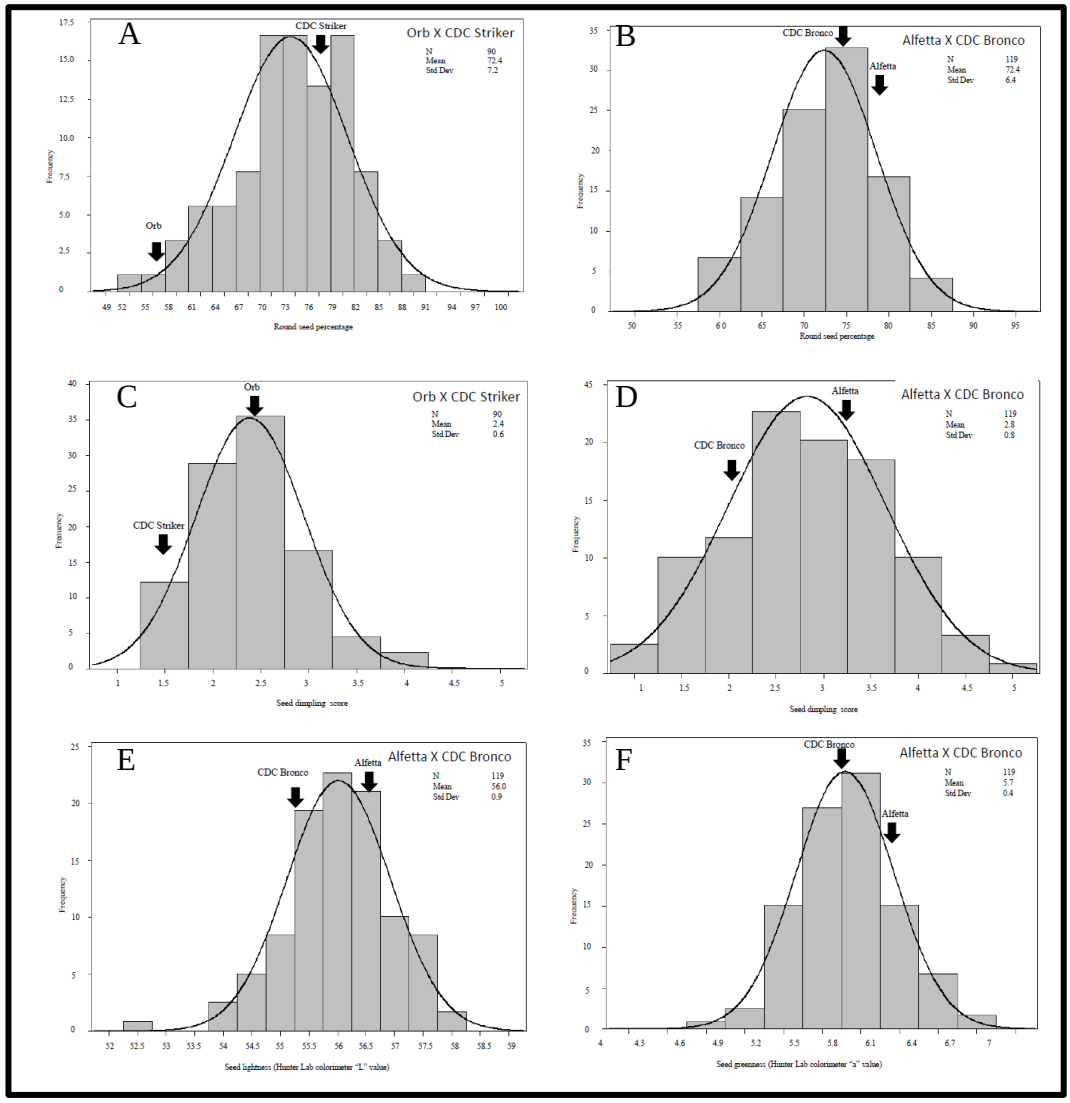

Fig. 4.1. Frequency distribution of the average phenotypic estimates over two years and two locations for the 120 RILs of Alfetta X CDC Bronco and 90 RILs of Orb X CDC Striker populations. The mean phenotypic values of the parental cultivars are shown with a black arrow. The black solid line of the histograms indicates the expected normal distribution curve.

Table 4.4. Correlation between visual quality trait estimates for 120 RILs of the Alfetta X CDC Bronco population over two years at two locations.

	Lightness	Greenness	Visual greenness	Seed shape	Seeddimpling
Lightness	-	-0.07^{NS}	-0.14 ^{NS}	-0.33**	0.32**
Greenness		-	-0.72***	0.07 ^{NS}	0.05 ^{NS}
Visual greenness			-	-0.31**	0.24**
Seed shape				-	-0.54***
Seed dimpling					-

Note: ^{NS}, not significant; *, significant at $P \leq 0.05$; ** , significant at $P \leq 0.01$; *** , significant at $P \leq 0.001$. For explanation of lightness, greenness, visual greenness, seed shape and seed dimpling, refer to Table 4.2.

The broad-sense heritability estimates for seed lightness, greenness, and visual greenness of the Alfetta X CDC Bronco population were 0.67, 0.63 and 0.70, respectively. These moderate heritability estimates indicate the substantial influence of environmental factors on expression of seed color in this yellow pea population. The heritability estimates for the Orb X CDC Striker and Alfetta X CDC Bronco population for dimpling were 0.60 and 0.86, respectively. Thus, genotype X environment factors were quite important in the Orb X CDC Striker population, whereas genetic contributions were more substantial in the Alfetta X CDC Bronco population.

4.4.2 Genetic linkage mapping and QTL analysis

4.4.2.1 Genetic linkage map of Alfetta X CDC Bronco population

Preliminary SSR primer screening identified 59 of the 350 primer pairs tested with polymorphic bands between Alfetta and CDC Bronco. Of the 59 polymorphic primer pairs, 24 were employed to genotype the 92 selected RILs resulting in the identification of 30 SSR loci. A total of 29 AFLP primer combinations were utilized to identify 274 polymorphic loci.

A genetic linkage map for the Alfetta X CDC Bronco population was constructed utilizing a total of 304 markers, but only 223 markers were mapped (27 SSR and 196 AFLP markers) (Fig. 4.2). Total coverage of the map was 450 centiMorgans (cM) and the average marker distance was 2.0 cM. Eleven linkage groups (LG) were identified at LOD value of 6 and anchored to the previously mapped seven linkage groups of the pea genome using the SSR markers described by Loridon et al. (2005). Of the 11 LGs, 9 were assigned to 6 (LGI, LG II, LG III, LG IV, LG VI, and LG VII) of the 7 LGs previously reported. Two LGs (LG E and LG F) were unassigned owing to lack of anchor markers mapped.

4.4.2.2 Genetic linkage map of Orb X CDC Striker population

The genetic linkage map of the Orb X CDC Striker population described by Ubayasena et al. (2010) was used in this study to identify the QTLs responsible for seed shape and dimpling. Alignment of both maps to construct a common consensus map was not possible owing to lack of common marker alleles.

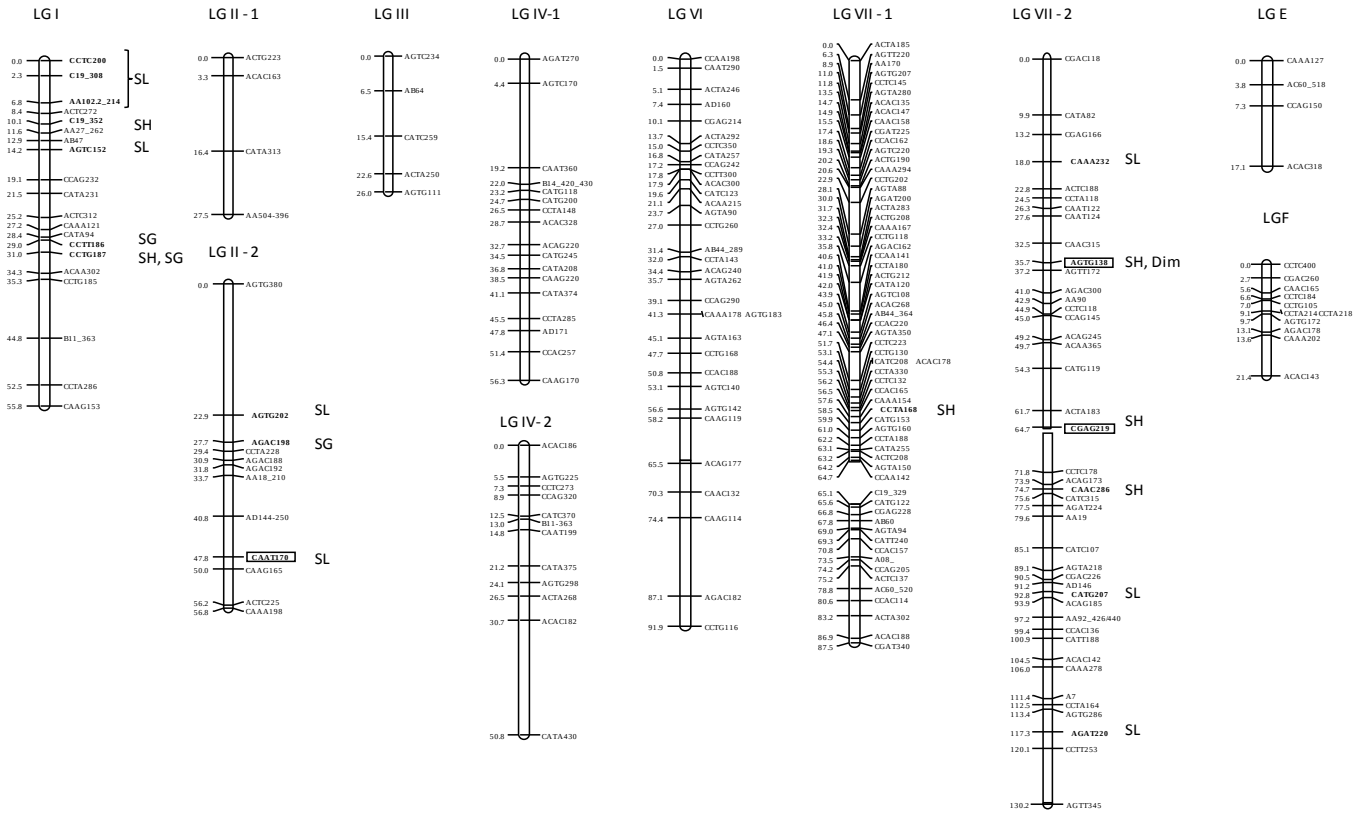

Fig. 4.2. Genetic linkage map based on AFLP and SSR marker segregation of 92 RILs of the Alfetta X CDC Bronco population. LG I, LG II, LG III, LG IV, LG VI, and LG VII represent the linkage groups assigned to the seven chromosomes recognized in pea genome. LG E and F are unassigned groups owing to lack of anchor markers. The genetic distances calculated in centimorgans (cM) are indicated to the left of each LG. Markers in bold are the closest markers for the QTL of interest. The trait associated with each QTL is given to the right side of the LG as SL (seed lightness), SG (seed greenness), Dim (seed dimpling) and SH (seed shape). QTLs identified in two or more environments are indicated with a box around the closest marker mapped.

4.4.2.3 QTL analysis

QTL regions associated with visual quality traits were located by interval mapping and confirmed by composite interval mapping using MapQTL software (Van Ooijen 2004). The significant threshold of LOD scores for QTLs was determined by a permutation test. Phenotypic estimates of the 90 RILs of the Orb X CDC Striker population were utilized to identify QTLs associated with seed shape and seed dimpling. In order to identify QTLs associated with seed shape, dimpling, and seed color in yellow peas, the phenotypic estimates based on the field experiments of 92 RILs selected from the Alfetta X CDC Bronco population were utilized. As LG V was not identified and LG III was not fully covered in the Alfetta X CDC Bronco map, and LG VI and LG VII were not fully covered in the Orb X CDC Striker map, detection of QTLs on those LG was hindered in this study. All the QTLs detected on these two linkage maps for the visual quality traits investigated in this study are given in Fig 4.3. Detailed description of all QTLs identified for seed color in yellow peas, shape and dimpling are given in Appendix 4, 5, and 6, respectively.

The QTLs associated with visual quality traits that were detected in at least two environments are given in Table 4.5. One QTL was detected on LG II-2 in 2006 at Rosthern and in 2006 at Saskatoon associated with an AFLP marker locus 'CAAT170' and explained 10.1% and 17.3% of the phenotypic variability of seed lightness in yellow peas, respectively. This allele was contributed by Alfetta to increase the lightness of the seeds by 0.42 (2006 Rosthern) and 0.56 (2006 Saskatoon).

Three QTLs associated with seed shape were detected on LG IV-2 (Orb X CDC Striker), LG VII-2 (Alfetta X CDC Bronco) and LG A (Orb X CDC Striker). The QTL on LG VII-2 was detected in 2006 and 2007 at Saskatoon and explained 14.7% and 25.0% of the total phenotypic variability, respectively. The allele associated with this QTL was the AFLP marker 'CGAG219' contributed by CDC Bronco to increase the round seed percentage by 2.9% (2006) and 3.6% (2007). The QTL on LG IV-2 was detected at Rosthern in both years and associated with the AFLP marker locus 'CCAG207' accounting for 17.0% (2006) and 6.5% (2007) of the total phenotypic variability. This allele was contributed by Orb to increase the round seed percentage by 4.6% (2006) and 2.2% (2007). The QTL detected on LG A was found at Rosthern and Saskatoon in 2007 explaining 14.2% and 9.6% of the total phenotypic variability, respectively.

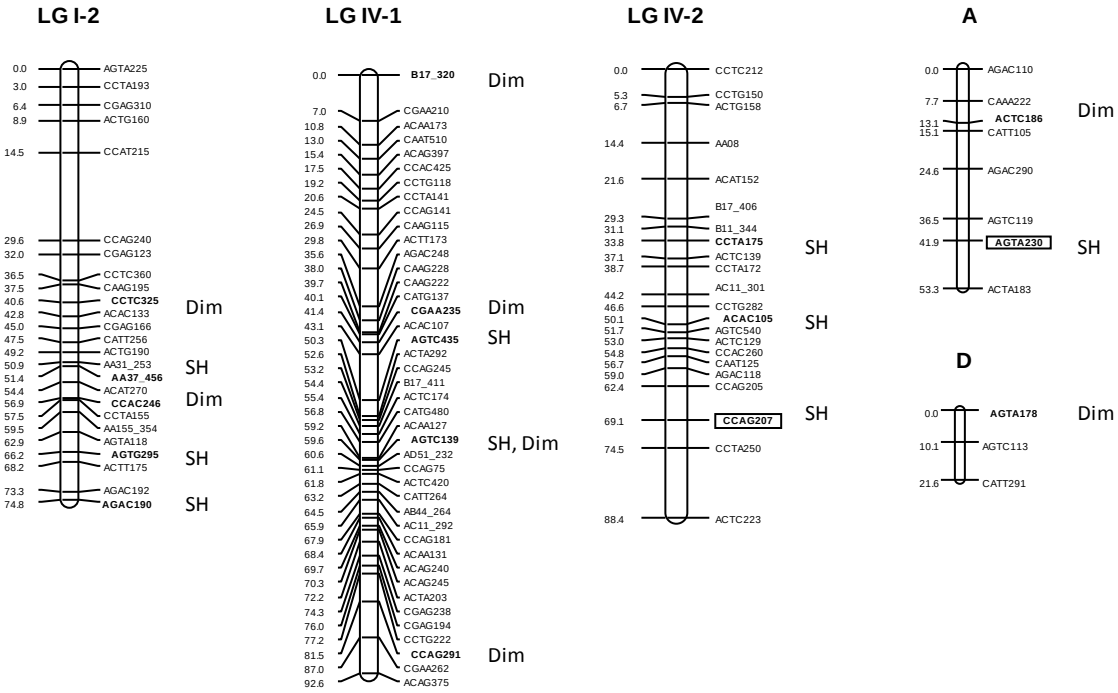

Fig. 4.3. Linkage groups illustrating QTL regions from the Orb X CDC Striker RIL population associated with seed shape and seed dimpling. LG I and IV represent chromosomes 1 and 2 of the pea genome. LG A and LG D are unassigned groups due to lack of anchor markers. The genetic distances calculated in centimorgans (cM) are indicated to the left of each LG. Markers in bold are the closest markers for the QTL of interest. The trait associated with each QTL is given to the right side of the LG as Dim (seed dimpling) and SH (seed shape). QTLs identified in two or more environments are indicated with a box around the closest marker mapped.

Table 4.5. QTLs for seed lightness, seed shape and seed dimpling identified in at least two environments in either pea RIL population Orb X CDC Striker or Alfetta X CDC Bronco.

Phenotype	Experimental sites	RIL population	LG	Closest Marker[a]	Location (cM)	r^2 (%)[b]
Lightness	2006 Rosthern and Saskatoon	Alfetta X CDC Bronco	LG II-2	CAAT170	43.8-47.7	13.7
Seed shape	2006 and 2007 Saskatoon	Alfetta X CDC Bronco	LG VII-2	CGAG219	64.7-66.7	19.9
Seed shape	2006 and 2007 Rosthern	Orb X CDC Striker	LG IV-2	CCAG207	69.0	11.8
Seed shape	2007 Rosthern and Saskatoon	Orb X CDC Striker	LG A	AGTA230	38.5-41.5	8.3
Seed dimpling	2006 Saskatoon and Rosthern, 2007 Rosthern	Alfetta X CDC Bronco	LG VII-2	AGTG138	33.5-36.7	14.5

[a]For detailed information, refer to Appendix 4, 5 and 6.

[b]Average phenotypic variation explained by the detected QTL.

The AFLP marker locus 'AGTA230' associated with this QTL was contributed by CDC Striker and increased the round seed percentage by 2.5% (Rosthern) and 2.6% (Saskatoon).

A QTL associated with dimpling was detected on the Alfetta X CDC Bronco genetic linkage map on LG VII-1 explaining 19.5%, 10.8% and 13.2% of the total phenotypic variability at Saskatoon (2006 and 2007) and Rosthern (2007) locations, respectively.
Except for the Saskatoon location in 2007, this QTL was significant at $P \leq 0.05$. The allelic contribution for this QTL was by CDC Bronco to increase the visual score by 0.6 (2006 Saskatoon), 0.4 (2007 Rosthern) and 0.3 (2007 Saskatoon). However, no QTLs detected on the Orb X CDC Striker genetic linkage map were detected in more than one environment.

The total phenotypic variability explained by all the detected QTLs for both locations over two years tested is summarized in Table 4.6. The total effect of QTLs detected for seed lightness in yellow peas ranged from 22.2% (2007, Saskatoon) to 38.5% (2006, Rosthern). The total phenotypic variability explained by the detected QTLs for greenness ranged from 0% (2007, Saskatoon and Rosthern) to 26.7% (2006, Saskatoon). Total phenotypic variability for seed shape explained by the QTLs identified in this study ranged from 22.9% (2007, Saskatoon for the Orb X CDC Striker population) to 49.6% (2007, Rosthern for the Orb X CDC Striker population). In contrast with the other two traits studied, QTLs identified for seed shape explained more than 20% of the phenotypic variability in both populations at both locations in both years. QTLs for seed dimpling were better resolved in the Orb X CDC Striker population compared to the Alfetta X CDC Bronco population. The amount of phenotypic variability explained by the QTLs identified in the Orb X CDC Striker population ranged from 14% (2006, Saskatoon) to 33.2% (2007, Saskatoon), whereas the QTLs detected for the Alfetta X CDC Bronco population explained 0% (2007, Rosthern) to 19.5% (2006, Saskatoon) of the phenotypic variability.

Table 4.6. Amount of phenotypic variability in seed visual quality traits explained by the detected QTLs for two locations over two years for the Orb X CDC Striker and Alfetta X CDC Bronco populations.

Phenotype	Population	Year	Location	Total r^2 (%)[a]
Lightness	Alfetta X CDC Bronco	2006	Rosthern	38.5
		2006	Saskatoon	24.4
		2007	Rosthern	22.2
		2007	Saskatoon	29.2
Greenness	Alfetta X CDC Bronco	2006	Rosthern	11.3
		2006	Saskatoon	26.7
		2007	Rosthern	00.0
		2007	Saskatoon	00.0
Seed shape	Alfetta X CDC Bronco	2006	Rosthern	24.9
		2006	Saskatoon	25.9
		2007	Rosthern	28.6
		2007	Saskatoon	25.0
Seed shape	Orb X CDC Striker	2006	Rosthern	26.3
		2006	Saskatoon	34.6
		2007	Rosthern	49.6
		2007	Saskatoon	22.9
Seed dimpling	Alfetta X CDC Bronco	2006	Rosthern	13.2
		2006	Saskatoon	19.5
		2007	Rosthern	00.0
		2007	Saskatoon	10.8
Seed dimpling	Orb X CDC Striker	2006	Rosthern	32.2
		2006	Saskatoon	14.0
		2007	Rosthern	29.3
		2007	Saskatoon	33.2

[a]The amount of phenotypic variation explained by the detected QTLs.

4.5 Discussion

To qualify for Canada No. 1 grade, yellow pea seeds must have a bright yellow appearance (Canadian Grain Commission, 2008), which is mainly attributed to the color of the cotyledons at maturity, but could also be influenced by the degree of translucency of the seed coat that affects the visibility of the underlying cotyledon color (McCallum et al. 1997). Some yellow pea cultivars have a tendency toward light brown or greenish seed coats because of the retention of some green pigments at maturity (Lamprecht 1959). The gene *gla* that conditions the seed coat characteristics of white-flowered pea genotypes to colorless versus greenish was reported by Lamprecht (1959).

Seed shape also plays an important role in determining the market value of field pea (McPhee 2007). Genetic estimates of seed shape have been challenging because of the subjectivity of assessment methods (Salas et al. 2006; Cober et al. 1997). Salas et al. (2006) estimated seed volume using seed width, seed length, and seed height of 25 seeds from each experimental unit to phenotype three RIL populations of soybean for seed shape, while Cober et al. (1997) used digital imaging to determine seed shape. The spherical-nonspherical seed sorter utilized in this study was effective in determining the round seed percentage of a representative seed sample for estimating the genetic parameters associated with seed shape.

In contrast with the already well-characterized wrinkled seed garden pea phenotype (Smith 1973; Hedley et al. 1986; Wang et al. 1990; Bhattacharyya et al. 1993;), dimpling of field pea "seeds with close sets of small shallow impressions on testa" as described on the Pisum Genetics Association web site (http://data.jic.bbsrc.ac.uk/cgi-bin/pgene/default.asp?ID=458) citing Lamprecht 1962) is an important visual quality trait for breeding. Lamprecht (1962) described the inheritance of seed dimpling as monogenic and the gene responsible as *mifo* located on LG II of the pea genome. The occurrence of pectic polysaccharide domains in cells and tissues of the testa plays an important role in maintaining the mechanical properties of developing pea seeds, especially at the later stages of development (McCartney and Knox 2002). Despite the simply inherited nature of *mifo*, field pea breeding observations suggested a quantitative inheritance pattern for this trait. Phenotypic evaluation of seed dimpling in this study was conducted by assigning a visual score from 1 to 5 considering the number of dimpled seeds

in a sample and the intensity of the dimples on dimpled seeds to quantify the effect of this trait in the market place.

This research suggests that the expression of *mifo* may be affected by epistatic interaction with other genes to condition the severity of seed dimpling in field pea. Furthermore, a significant ($P \leq 0.001$) negative correlation was observed between dimpling and seed shape (i.e., round seeds had fewer tendencies to dimpling, which is favorable for breeding for human consumption markets). The observed moderate heritability estimate for the Orb X CDC Striker population and high heritability estimate for the Alfetta X CDC Bronco population for seed dimpling further confirmed the influence of environmental factors and genotype X environmental interaction on the expression of this trait. Therefore, evaluation of segregating populations in different environments at later stages of the selection program is recommended.

The continuous distributions observed for seed color, seed shape and seed dimpling indicated that the genetic control of these traits are quantitative, complex and controlled by many loci. Both populations showed transgressive segregation for the visual quality traits studied, indicating the potential of genetic improvement through breeding. The occurrence of transgressive segregation could be explained by the combination of favorable or unfavorable alleles with additive effects contributed by both parents (Orf et al. 1999; Salas et al. 2006). This also highlighted the potential benefits of molecular markers to screen parental cultivars for the presence of favorable and unfavorable alleles.

The heritability estimates of seed lightness and greenness indicated that the phenotypic variability of yellow seed color was highly influenced by environmental factors. Thus, selection for bright yellow color should be delayed to a generation in which sufficient seeds and a manageable number of breeding lines have been achieved for intensive evaluations in multi-location, replicated field experiments. This study indicated that the inheritance of seed shape is quantitative and under polygenic control. These findings are in agreement with soybean studies (Cober et al. 1997; Salas et al. 2006). High heritability estimates of seed shape in both RIL populations indicated that the genetic contribution for the determination of seed shape is high. In addition, the use of molecular markers linked with the alleles associated with the control of seed shape to select parents and breeding lines is recommended.

The length of the genetic linkage map for the Alfetta X CDC Bronco RIL population is less than previously published for pea, which ranged from 1104 cM to 2416 cM (McCallum et al.

1997; Laucou et al. 1998; Irzykowska et al. 2001; Tar'an et al. 2003; von Stackelberg et al. 2003; Tar'an et al. 2004; Loridon et al. 2005; Aubert et al. 2006). One possible reason could be the extent of marker polymorphism in this population. Initial parental screening experiments revealed low level polymorphism for SSR primers (19%) and AFLP primers (nine loci per AFLP primer pair). Difficulties in covering the whole barley genome with a low level of marker polymorphism were discussed by Li et al. (2008). In addition, the limited map coverage obtained when using SSR and AFLP markers alone in pea genetic linkage mapping was discussed in Ubayasena et al. (2010). Development of more robust SNP markers could greatly enhance the ability to cover the whole genome and increase the density of the pea genetic linkage map (Hyten et al. 2004). The incorporation of SNP markers will also facilitate the construction of a more informative consensus linkage map by joining the linkage maps for both pea populations.

Despite the low genome coverage of the linkage map of the Alfetta X CDC Bronco population, eight QTLs associated with seed lightness and three QTLs associated with seed greenness were identified in this study. These QTLs indicated that LG I, LG II and LG VII were most important for controlling seed color related visual quality traits. Of the 10 QTL regions associated with seed color of yellow peas identified in this study, 9 were identified from only one environmental condition. Thus, these QTLs were highly dependent on environmental factors. The low level heritability estimates of the seed-color-related measurements further validate this observation. However, the first 6.8 cM region of LG I appears to be important in controlling seed color in yellow pea. This QTL region was detected in all environments tested with different magnitudes and associated with different marker alleles. Previous publications have reported on genetic analysis and QTL mapping related to seed color in green cotyledon pea (McCallum et al. 1997; Ubayasena et al. 2010); this is the first study we are aware of to investigate the genetics and associated QTLs in yellow cotyledon pea.

Almost all the seed-shape-related studies in pea to date have characterized the molecular and biochemical differences between round and wrinkled type seeds (Hedley et al. 1986; Wang et al. 1990; Bhattacharyya et al. 1993). To our knowledge this is the first study reporting QTLs associated with seed shape in field pea. Six QTLs from Alfetta X CDC Bronco RIL population were detected on LG I and LG VII. For the Orb X CDC Striker population, nine QTL regions were identified on LG I, LG IV and unassigned LG A. However only three QTLs, one on Alfetta X CDC Bronco (LG VII-2) and two on Orb X CDC Striker (LG IV-2 and LG A), were detected

in at least two environments, indicating that these three QTLs could be given priority in breeding programs.

Timmerman-Vaughan et al. (1996) reported several QTLs associated with seed weight of pea on LG III, IV and V in the 'Primo' X 'OSU442-15' cross and on LG I, III and VII in the 'JI1794' X 'Slow' cross. The QTLs reported in this study associated with seed shape were also mapped mainly on LG I, IV, VII and unassigned LG A indicating the importance of these LGs for the expression of seed quality characteristics. Precise comparisons of the QTLs identified in this study to reported seed weight associated QTLs by Timmerman-Vaughan et al. (1996) is not possible due to the differences in the marker systems used.

Only one QTL region was identified for the Alfetta X CDC Bronco population associated with dimpling on LG VII in three of the four experimental environments tested in this study. This QTL explained 10.8% (2007, Saskatoon) to 19.5% (2006, Saskatoon) of the phenotypic variability. In contrast, eight QTLs were detected for the Orb X CDC Striker population on LG I, LG IV, LG A and LG D. However none of these QTLs were consistent over the tested environments. This study did not detect any QTLs regions on LG II where *mifo* is located. This could be due to marker scarcity on LG II in both genetic maps, or all parents carrying the same allele at this locus. The amount of phenotypic variability explained by the detected QTLs ranged from 14% (2006 Saskatoon) to 32.2% (2006, Rosthern), indicating the importance of further characterization of the dimpling trait in field pea.

CHAPTER 5

5. Gene expression profiles of seed coats and biochemical properties of seed coats and cotyledons of two field pea (*Pisum sativum*) cultivars contrasting in green cotyledon bleaching resistance

5.1 Abstract

Visual quality is one of the major factors determining the market value of field pea (*Pisum sativum* L.). Breeding for improved visual quality of pea seeds is currently a challenging task, mainly because of the complexity and the lack of sound genetic knowledge of the traits. The objectives of this research were to characterize post-harvest cotyledon bleaching resistance in green pea at the biochemical and gene expression levels. Seed coats and cotyledons of two pea cultivars, CDC Striker (bleaching resistant) and Orb (bleaching susceptible) at three developmental stages (14, 21 and 28 days after flowering (DAF)) and following exposure to accelerated bleaching conditions after harvest (0 (35DAF), 3, 6, and 13 days after bleaching (DAB) were evaluated. CDC Striker had a slower rate of chlorophyll degradation in cotyledons, and a higher total carotenoids to chlorophyll ratio in seed coats, than Orb when seed samples were exposed to high intensity light. An oligo-nucleotide microarray (Ps6kOLI1) revealed that gene expression profiles of the CDC Striker and Orb seed coats were significantly different during seed developmental stages. A significant up regulation of genes involved in the production and accumulation of secondary metabolites responsible for antioxidant properties including epiafzelechin, epcatechin, epigallocatechin, kaempferide, kaempferol 3-O-β-D-sophorotrioside, O-quercetin and rutin, in the seed coats of CDC Striker were observed. Thus, bleaching resistance in field pea could be due to the accumulation of specific carotenoids and phenolic compounds which quench excess light or scavenge free radical singlet oxygen molecules. The candidate genes identified in this project could be used for the development of gene specific markers after further validation.

Key words: field pea, bleaching, pigments, gene expression, cDNA, microarray

5.2 Introduction

Substantial economic losses due to down-grading of green field pea (*Pisum sativum* L.) based on natural green color degradation, typically referred to as bleaching, is one of the main constraints faced by pea producers and traders worldwide (Shepherd, 1959; McCallum et al. 1997; Cheng et al. 2004). Bleaching is due to genetic and/or environmental factors during seed maturation and post-harvest storage which lead to degradation of chlorophyll pigments from the green cotyledon tissues (Lamprecht, 1959; Maguire et al. 1973; Dribnenki, 1979; McCallum et al. 1997; Canadian Grain Commission, 2008). In addition to down-grading, adverse physiological effects due to bleaching, such as poor germination and loss of early seedling vigor have been reported (Maguire et al. 1973; Loria, 1979).

Accelerated bleaching was observed when seeds were exposed to light and seed moisture contentration exceeding 20% (Riehle and Muelbauer, 1975). Gubbels and Ali-Khan (1990) described a strong negative correlation between hard seeds and bleaching rate. Ubayasena et al. (2010) reported that the seed coat was the tissue which protected cotyledons from bleaching. Physical and chemical properties acquired by the seed coat during seed development and maturation could provide varying levels of green color protection in field pea genotypes. McCallum et al. (1997) found that the rate of chlorophyll degradation during seed maturation in the bleaching resistant cultivar 'Promo' was significantly lower than that of the bleaching susceptible cultivar 'OSU442-15'. Despite studying the pigment composition of whole seeds, the pigment dynamics of seed coats and cotyledons during seed development, maturity and post-harvest bleaching were not addressed by McCallum et al. (1997). Cheng et al. (2004) demonstrated that bleaching in dry peas exposed to light was not an enzyme dependant reaction, but rather due to loss of chlorophyll protective mechanisms. Furthermore, the biochemical properties of the seed coats during seed development and bleaching periods have not been addressed to date.

In addition to the biochemical properties, gene action and transcriptional regulation in seed coats during seed development may be informative in understanding the underlying bleaching resistance mechanism in seed coats. Gene expression profiles of the model legume crop *Medicago truncatula* using cDNA micro- and macro-array studies revealed genes responsible for nodulation and arbuscular mycorrhiza development due to colonization of *Glomus intraradices* (Küster et al. 2004). They reported several root nodule-specific genes

responsible for the development and functioning of the endosymbioses relationships in legume plants. El Yahyaoui et al. (2004) unravelled the complex symbiotic relationship in nitrogen fixing bacterium (*Sinorhizobium meliloti*) using micro- and macro-array analysis. Gene expression analysis of pea embryos using a cDNA microarray consisting of 5548 seed-specific genes suggested that the repression of sucrose nonfermenting-1-related protein kinase gene caused some functional irregularities similar to abscisic acid (ABA) insensitivity (Radchuk et al. 2006).

An oligo-nucleotide microarray (Ps6kOLI1) consisting of 5220 Expressed Sequence Tags (EST) from pea cotyledons and seed coats was developed by the Grain Legumes Technology Transfer Platform (GL-TTP) of the European Union Grain Legumes Integrated Project (GLIP) (Küster and Dondrupup, 2006). This microarray consisted of 5220 70-mer oligonucleotide probes, and each probe was printed in triplicate with other quality control probes including empty spots within each microarray (Kathleen et al. 2008). The availability of a pea seed specific cDNA microarray was recognized as an important tool to address questions related to gene expression in developing seeds (Küster and Dondrup, 2006).

Hence the objectives of this study were, (1) to investigate the gene expression profiles of field pea seed coats at three developmental stages (14, 21 and 28 days after flowering) in bleaching resistant (CDC Striker) and bleaching susceptible (Orb) green pea cultivars, to understand the genomic regulation of the bleaching resistance phenotype during seed development and (2) to correlate transcriptional changes in seed coats with changes in seed coats and cotyledons biochemical properties with respect to Chlorophyll-a (Chl-a), Chlorophyll-b (Chl-b), total chlorophyll, and total carotenoids during seed development and accelerated bleaching conditions.

5.3 Materials and Methods

5.3.1 Plant materials

Two green cotyledon type field pea cultivars, ‘CDC Striker’ developed by Warkentin et al. (2004) at the Crop Development Centre, University of Saskatchewan, with good bleaching resistance and ‘Orb’ developed by Sharpes International, UK, with poor bleaching resistance, were used. Plants were grown in a growth chamber of the phytotron facility at the University of

Saskatchewan, Canada in 2007, in two gallon plastic pots were filled with Sunshine mix 4 (Sun Gro Horticulture Canada Ltd., Seba Beach, AB, Canada). The experiment consisted of two biological replicates, with one replicate grown at a time. In order to collect enough seed material for biochemical and gene expression analysis, 32 plants of each cultivar per replicate were grown. Each replicate consisted of 16 pots with two plants per pot. Pots were rearranged within the growth chamber every second day to ensure the plants were exposed to unbiased growing conditions. The growth chamber was set to provide 18 ^{o}C night time and 23 ^{o}C day time temperatures with 16 hour day length. The chamber was illuminated during the day time with incandescent and fluorescent lights to provide light intensity of 400 µmol $m^{-2}s^{-1}$. Plants were watered regularly until the last pods turned brown.

In addition to the growth chamber experiment, seed samples of these two cultivars, and selected RILs of the Orb X CDC Striker mapping population described in Ubayasena et al. (2010) were obtained from field experiments conducted at two locations in southern Saskatchewan (Saskatoon and Rosthern) in 2006 and 2007. Seeds of five bleaching resistant and five bleaching susceptible RILs were selected.

5.3.2 Sampling and preparation of seed tissues

Flowers at the second, third and fourth reproductive internodes were tagged when they first opened with dated tags. Several pods were harvested at 14, 21, 28 and 35 days after flowering (DAF) and seeds were immediately dissected to separate seed coats and cotyledons. Five subsamples of cotyledons and seed coats were made for RNA extraction (1 subsample), pigment analysis (3 subsamples) and dry weight determination (1 subsample). The seeds sampled at 35 DAF were physiologically mature with desiccated seed coats and; therefore, these seeds were only used in the pigment analysis.

Tissues collected for RNA extraction were immediately frozen in liquid nitrogen and ground using a mortar and pestle and the ground tissue was collected into a 15 mL tube and stored at -80 ^{o}C until extraction. Three subsamples collected for pigment analysis were collected into 15 mL tubes, weighed and immediately frozen in liquid nitrogen before storage at -80 ^{o}C until analysis. The subsample of each tissue collected for dry weight determination was oven dried at 60 ^{o}C for 24 hours to determine the dry weight at each sampling point.

All the remaining pods were harvested at 35 DAF, threshed and divided into 4 subsamples containing 15 seeds each in transparent bags (Nasco Whirl-pak, Fort Atkinson, WI, USA) to study their bleaching resistance with artificial light. Holes were made in these plastic bags using a 3 mm needle to allow the air movement. Bags were randomly spread in a growth chamber set to deliver optimized conditions for accelerated bleaching, as described in Ubayasena et al. (2010). Subsamples representative of both replicates of each cultivar were collected and prepared for pigment extraction at 3, 6, and 13 days after exposure to accelerated bleaching conditions (DAB). All the seed subsamples collected at different bleaching stages including 35DAF (0DAB) were dehulled using a Satake Grain Testing Mill model TM05 (Satake Corporation, Taitoku, Tokyo, Japan) and cotyledons and seed coats were collected separately. The cotyledons and seed coats collected from dried seed tissues were ground using a Udy sample mill (Udy Co. Fort Collins, CO, USA) equipped with 0.5 mm mesh and stored at -20 ^{o}C until analysis.

Ninety-six mature seed samples of 10 RILs and two parental cultivars (12 lines X 2 replicates X 2 locations X 2 years) obtained from the field experiments were dehulled and seed coats were ground as explained above and stored at -20 ^{o}C until analysis.

5.3.3 Extraction and determination of pigments from seed tissues

Seed coats and cotyledons from each developmental stage were analyzed separately for pigment profiles (Chl-a, Chl-b and total carotenoids). Extraction and analysis of these pigments were conducted by spectrometric techniques as described by Sims and Gamon (2002). Pigments were extracted from the collected tissue samples using cold acetone/0.1M Tris buffer solution (80:20 vol:vol, pH=7.8). Immature seed tissues with known fresh weights were used without grinding to extract pigments. Four milliliters of Tris buffered acetone was added to the tubes and shaken for 48 hours at 4 ^{o}C in the dark. Tubes were centrifuged at 8,000 x g for 5 min and 1 mL of supernatant was assessed in disposable UV visible cuvettes (1 cm path length) and absorbance was measured at 326, 433, 470, 537, 647, 663 and 720 nm and recorded using a HP 8453 (Agilent Technologies Canada Inc. Mississauga, ON, Canada) diode array spectrophotometer. The samples were re-extracted by adding 4 mL of Tris buffered acetone and the absorbance was recorded and the estimated pigment concentrations were added to the initial readings. For mature seed tissues 4 mL of Tris buffered acetone was added to 1 mg of ground tissue then shaken for

48 hours at 4 ^{o}C at dark and centrifuged at 8,000 x g for 5 min. One millilitre of the supernatant was used to measure the absorbance using the spectrophotometer. Only one extraction was carried out for the ground tissue as preliminary experiments showed trace amounts of pigments left after the first extraction (data not shown).

Chl-a, Chl-b and total carotenoids concentrations of the samples were determined using the equations (1), (2), (3) and (4) described by Sims and Gamon (2002).

$$\text{Anthocyanin} = 0.08173A_{537}-0.00697A_{647}-0.002228A_{663} \quad (1)$$

$$\text{Chl-a} = 0.01373A_{663}-0.000897A_{537}-0.003046A_{647} \quad (2)$$

$$\text{Chl-b} = 0.02405A_{647}-0.004305A_{537}-0.005507A_{663} \quad (3)$$

$$\text{Total carotenoids} = (A_{470}-(17.1 \text{ X (Chl-a + Chl-b)} - 9.479 \text{ X Anthocyanin}))/119.26 \quad (4)$$

Where A_x is the absorbance of the supernatant at wavelength X.
The units of the pigment concentration estimated using the above equations are micromoles per millilitre (µmol mL^{-1}) and converted to mg/100 g of dry matter basis using the mole units for Chl-a, Chl-b and total carotenoids (Lichtenthaler, 1987).

5.3.3.1 Data analysis

A multivariate analysis approach described by Rowell and Walters (1976) and Gurevitch and Chester (1986) was employed to investigate the trends in pigment concentrations over time. The multivariate analysis, ANOVA and correlations were performed using SAS software V8.0 (SAS Institute Inc., Cary, NC). LS means estimated by SAS were used to create graphs in Microsoft EXCEL software.

5.3.4 Total RNA extraction

Total RNA was extracted from the ground seed coat samples using the phenol:guanidine extraction protocol described by Ganeshan et al. (personal communication and later published in 2010), with minor modifications. Fifty milligrams of ground seed coat tissue was mixed with 6 mL of pre-warmed (65 ^{o}C) extraction buffer (100 mL of extraction buffer contained 50 mL of acidic phenol, 30 g guanidine HCL, 5 g of SDS, 5 mL of glycerol and 45 mL of RNAse free

water) in a 15 mL tube and incubated for 10 min at 65 ^{o}C. Tubes were mixed several times during incubation and then the supernatant was transferred to a new RNAse-free tube after centrifuging at 2000 x g rpm for 5 min at 4 ^{o}C. Tubes were mixed by vortexing for 15 s after adding 3 mL of chloroform and centrifuged at 14,000 x g at 4 ^{o}C. The top aqueous layer was transferred to new RNAse free tubes and the chloroform purification step was repeated twice. One milliliter of isopropanol was added to the transferred aqueous layer after final chloroform extraction and incubated at -80 ^{o}C for 15 min. The RNA was pelleted by centrifuging the tubes at 14,000 x g for 10 min at 4 ^{o}C. The RNA pellet was then washed twice with 75% aqueous ethanol, the supernatant was discarded, air dried and re-suspended in 100 µL of RNAse-free ultra pure water. The extracted RNA was then treated with TURBO DNase as described in the TURBO DNA-free protocol supplied by the manufacture (Applied Biosystems Inc. Foster City, CA. USA) to remove any contaminating genomic DNA. DNAase treated RNA was further purified using an Invitrogen total RNA extraction kit (Invitrogen, Burlington, ON, Canada) following the manufacturer's protocol. The quality and integrity of RNA was determined by running an aliquot on formaldehyde agarose gel (1% w/v) and the quantity was determined spectrophotometrically (Sambrook and Russel 2001). Purity of the RNA was determined by observing the ratio of absorbance values of 260 nm and 280 nm and the quantity was determined by the absorbance at 260 nm.

5.3.5 cDNA synthesis and labeling

Fluorescently labeled (Cy-3 or Cy-5) targets of single strand cDNA was synthesized using 20 µg of total RNA extracted from respective hybridization tissues (using one dye to label cDNA strand synthesized from one tissue type, Fig.5.1) using CyScribe post labeling kit (GE Healthcare Bio-Sciences, Uppsala, Sweden) following the manufacture's instructions. Synthesis of Cy-3 and Cy-5 labeled cDNA samples were verified by loading 2 µL aliquots mixed with 80% glycerol on 0.8% agarose (w/v) gels.

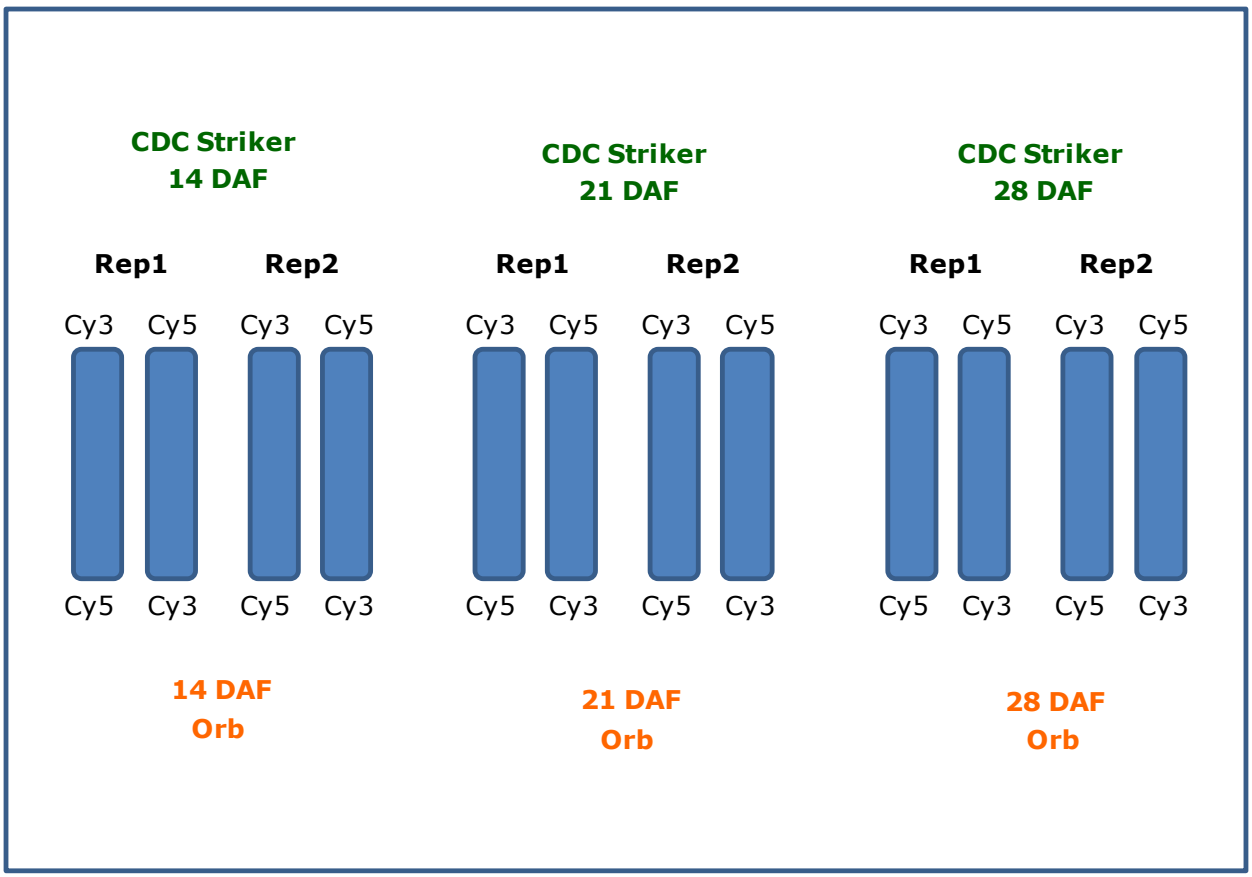

Fig. 5.1. Micro-array hybridization scheme used to investigate seed coat transcriptional profiles between CDC Striker and Orb. Cy3 and Cy5 indicate the dye color used to label cDNA synthesized from the RNA extracted from seed coat tissues collected at each developmental stage.

The gels were visualized using a Typhoon Scanner (GE Healthcare Bio-Sciences, Uppsala, Sweden) after electrophoresis for 20 min at 80 V in 1X TBE buffer (Fig. 5.2) at 633 nm to detect the red, Cy-5 and 532 nm to detect the green, Cy-3 labeled target cDNA. Equal volumes (60 µL each) of labeled cDNA from both tissue types used to hybridize on one microarray were combined in a dark 1.5 mL tube and dried down using a SpeedVac (Thermo Scientific, Asheville, NC. USA).

5.3.6 Ps6kOLI1 microarray

An oligo-nucleotide microarray (Ps6kOLI1) developed under the Grain Legumes Technology Transfer Platform (GL-TTP) of the Grain Legumes Integrated Project (GLIP) was utilized in collaboration with Dr. Helge Kuster (University of Bielefeld, Germany). This microarray consisted of 5220, 70-mer oligonucleotide probes, with each probe printed in triplicate, with other quality control probes including empty spots within each microarray (Kathleen et al. 2008). The array description file for Ps6kOLI1 microarray is located in the ArrayExpress database under the accession number A-MEXP-142 (http://www.ebi.ac.uk/microarray/).

5.3.6.1 Pre-hybridization processing of microarray slides

Immediately prior to the hybridization of labeled cDNA probes, Ps6kOLI1 microarray slides printed on Nexterion Slide E (Schott, Louisville, KY, USA) were pre-hybridized following the manufacturer's recommendation. Microarray slides were placed in plastic racks to avoid scratching the probe printed surface and to facilitate transfer from blocking and rinsing solutions. Slides were pre-washed in rinsing solution 1 (250 mL MilliQ water and 250 µL of Triton X 100 dissolved at 80 ^{o}C for 5 min and cooled to room temperature) for 5 minutes, rinsing solution 2 (500 mL MilliQ water and 50 µL 32% HCL) for 2 minutes, rinsing solution 3 (225 mL MilliQ water and 26 mL of KCL) for 10 minutes, and washed for 1 minute in 250 mL of MilliQ water. The pre-washed slides were then transferred immediately to pre-warmed (50 ^{o}C) blocking solution (150 mL MilliQ water, 47 µL 32% HCL and 50 mL 4X blocking solution (Schott, Louisville, KY, USA)) in a glass container. After incubation in the blocking solution for 15 minutes, slides were washed in MilliQ water at room temperature and then dried by centrifuging at 480 x g for 3 min.

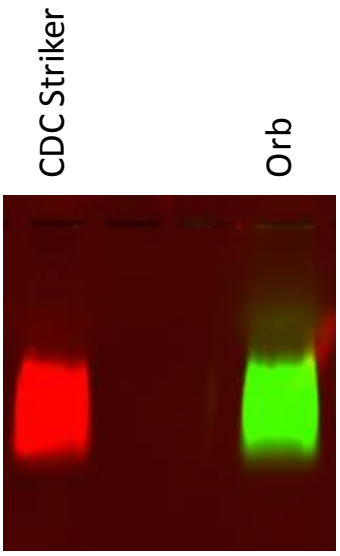

Fig.5.2. Gel image of synthesized Cy3 and Cy5 labeled single strand cDNA prior to micro-array hybridization.

5.3.6.2 Hybridization and acquisition of images

Completely dried labeled probe mix (Cy3 and Cy5) was re-suspended in 60 µL of DIG Easy Hyb (Roche Diagnostics, Laval, QC, Canada) and 5 µg of sonicated salmon sperm DNA (Invitrogen, Burlington, ON, Canada) and incubated for 5 min at 65 ^{o}C immediately before hybridization. The pre-hybridized micro-array slides were pre-warmed to 42 ^{o}C in an Arrayit hybridization chamber (Arrayit Corporation, Sunnyvale, CA, USA). The denatured probe mix was applied to the center and covered with a clean cover slip without incorporation of air bubbles. The hybridization chamber was sealed properly and incubated at 42 ^{o}C for 15 hours. After hybridization, microarrays were washed two times in 0.2 X SSC, 0.1% (W/V) SDS at room temperature for 2 min followed by two wash steps in 0.2 X SSC at room temperature. The final wash step was performed in 0.05 X SSC at 21 ^{o}C for 1 min, the slides were then dried by centrifuging at 480 x g for 3 min. All these steps were done in dark chambers to avoid exposure of the Cye dyes to light and bleaching. The dried slides were scanned using a GenePix microarray scanner (Molecular Devices, Sunnyvale, CA, U.S.A.) using a pixel size of 10 µm at 633 and 532 nm wave lengths with optimum laser strengths depending on the intensity of the labeled probes on the slides. Image files were stored as single TIFF image files. Duplicate hybridizations were performed by swamping the dye labels to eliminate the dye-related signal correlation bias.

5.3.6.3 Analysis of microarray data

Image analysis including spot finding, flagging bad spots due to hybridization artifacts, flagging spots based on the background intensity, and background subtraction of spots within each array were performed using the GenePix® Pro 6 software (Molecular Devices, Sunnyvale, CA, USA). After images were processed the data files were extracted in Gene Pix data output file format (.GPR). Data files were then uploaded to an ArrayLims microarray data management platform and imported to EMMA 2.8.2 microarray analysis software to evaluate transcriptional differences (Dondrup et al. 2003). Each microarray data set was filtered to remove the spots with intensity signal >55% of background + 1 standard deviation (SD) and with less than 3% pixel saturation for both 633 and 532 nm channels before normalization (Lowess normalization) for all the arrays hybridized. Differentially expressed genes were identified for each developmental stage using a Student t-test. Genes with $P \leq 0.05$ and M-value ($\log_2$ base ratio of the intensities

corrected to $\log_2$ overall intensity) ≥ 1.0 were identified as up regulated in CDC Striker, and genes with $P \leq 0.05$ and M-value ≤ -1.0 were identified as down regulated in CDC Striker compared to Orb. Gene expression over different developmental stages was examined by performing hierarchical clustering algorithms integrated into the EMMA 2.8.2 software (Dondrup et al. 2003). Finally, visualization of genes differentially regulated in CDC Striker and Orb at three developmental stages was linked with the respective functional biosynthesis pathways by connecting the annotated gene expression data obtained from the microarray hybridization to the GenDB genome annotation system by KEGG pathway analysis (Kyoto Encyclopedia of Genes and Genomes).

5.4 Results

5.4.1 Biochemical profiles of seed coats and cotyledons

Dry matter accumulation of the cotyledons and seed coats of Orb and CDC Striker showed a similar trend of increasing during the seed filling period and becoming stable by maturity (Fig. 5.3). Significant cultivar differences were observed for both cotyledons and seed coats at 14 and 28 days after flowering, where Orb seed coats had less dry matter (more moisture) than CDC Striker. This graph also indicated that the seed moisture percentage dropped to approximately 10% in both seed coats and cotyledons at 35 days after flowering and then remained at approximately this level during the post-harvest accelerated bleaching period.

Analysis of variance of the pigment concentration of cotyledons and seed coats of CDC Striker and Orb during seed development and accelerated bleaching periods are summarized in Tables 5.1 and 5.2, respectively. Dynamics of the pigments in cotyledons and seed coats during the seed development and seed bleaching periods of the two cultivars are illustrated in Fig.5.4 to 5.8. No significant differences for Chl-a, b and total chlorophyll concentration of the cotyledons were observed between the two cultivars at 14 and 21 DAF, but significant differences ($P \leq 0.05$) were observed at 28 and 35 DAF and during the accelerated bleaching period. The Chl-a, Chl-b and total chlorophyll concentrations were higher in Orb cotyledons compared to CDC Striker at 28 and 35 DAF.

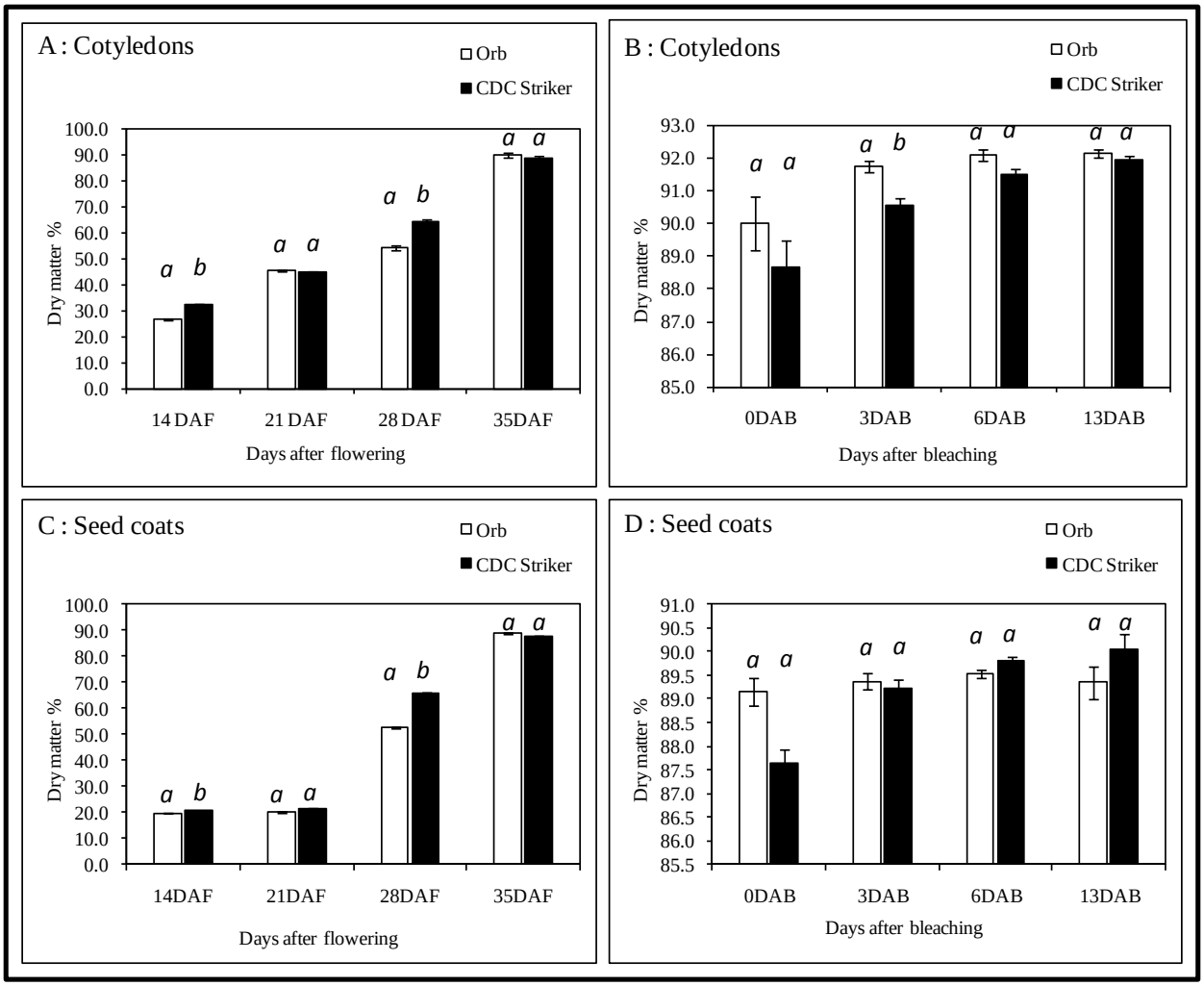

Fig. 5.3. Changes in percent dry matter of the cotyledons (A and B) and seed coats (C and D) of two pea cultivars (Orb and CDC Striker) during seed development (A and C) and light mediated bleaching (B and D) periods. Values with the same letters indicate no significant difference at based on the LSD $P \leq 0.05$. Error bars on the graphs indicate ± standard error.

Table 5.1. Mean squares of the pigment concentrations (mg/100 g of dry matter) of cotyledons of Orb and CDC Striker during seed development and accelerated bleaching.

Pigment	Mean squares						
	14DAF[†]	21DAF[†]	28DAF[†]	35DAF[†,#]	3DAB[§]	6DAB[§]	13DAB[§]
Chl-a	148.99 [NS]	0.43 [NS]	41.74*	5.81*	6.71*	3.65**	2.61**
Chl-b	13.88 [NS]	0.62 [NS]	6.25*	2.54*	0.16 [NS]	1.17**	1.07**
Total Chlorophyll	253.83 [NS]	2.07 [NS]	80.30*	16.04*	12.20**	8.96**	7.02**
Chl-a/b ratio	0.00 [NS]	0.08 [NS]	0.03 [NS]	0.08*	0.33*	0.00 [NS]	0.00 [NS]
Total carotenoids	32.30*	1.25[NS]	6.40*	0.53*	0.21*	0.37*	0.33*

Note: [†], days after flowering; [#], harvesting time (0 days after bleaching); [§],days after exposure to accelerated bleaching conditions; [NS], not significantly different between two cultivars at $P \leq 0.05$; [*], significantly different between two cultivars at $P \leq 0.05$; [**], significantly different between two cultivars at $P \leq 0.01$.

Table 5.2. Mean squares of the pigment concentrations (mg/100 g of dry matter) of seed coats of Orb and CDC Striker during seed development and accelerated bleaching.

Pigment	Mean square at each developmental and bleaching stage						
	14DAF[†]	21DAF[†]	28DAF[†]	35DAF[†,#]	3DAB[§]	6DAB[§]	13DAB[§]
Chl-a	0.23 [NS]	2.00 [NS]	127.26*	9.07**	0.26 [NS]	0.70**	0.48**
Chl-b	1.84 [NS]	0.35 [NS]	21.32*	4.45*	0.33*	0.70**	0.51**
Total Chlorophyll	3.37 [NS]	4.02 [NS]	252.76*	26.23**	1.19 [NS]	2.81**	1.97**
Chl-a/b ratio	0.50 [NS]	0.00 [NS]	0.12 [NS]	0.02 [NS]	0.01 [NS]	0.00 [NS]	0.04 [NS]
Total carotenoids	5.47**	0.50 [NS]	19.13**	1.47**	0.01 [NS]	0.17 [NS]	0.07*

Note: [†], days after flowering; [#], harvesting time (0 days after bleaching); [§],days after exposure to accelerated bleaching conditions; [NS], not significantly different between two cultivars at $P \leq 0.05$; [*], significantly different between two cultivars at $P \leq 0.05$; [**], significantly different between two cultivars at $P \leq 0.01$.

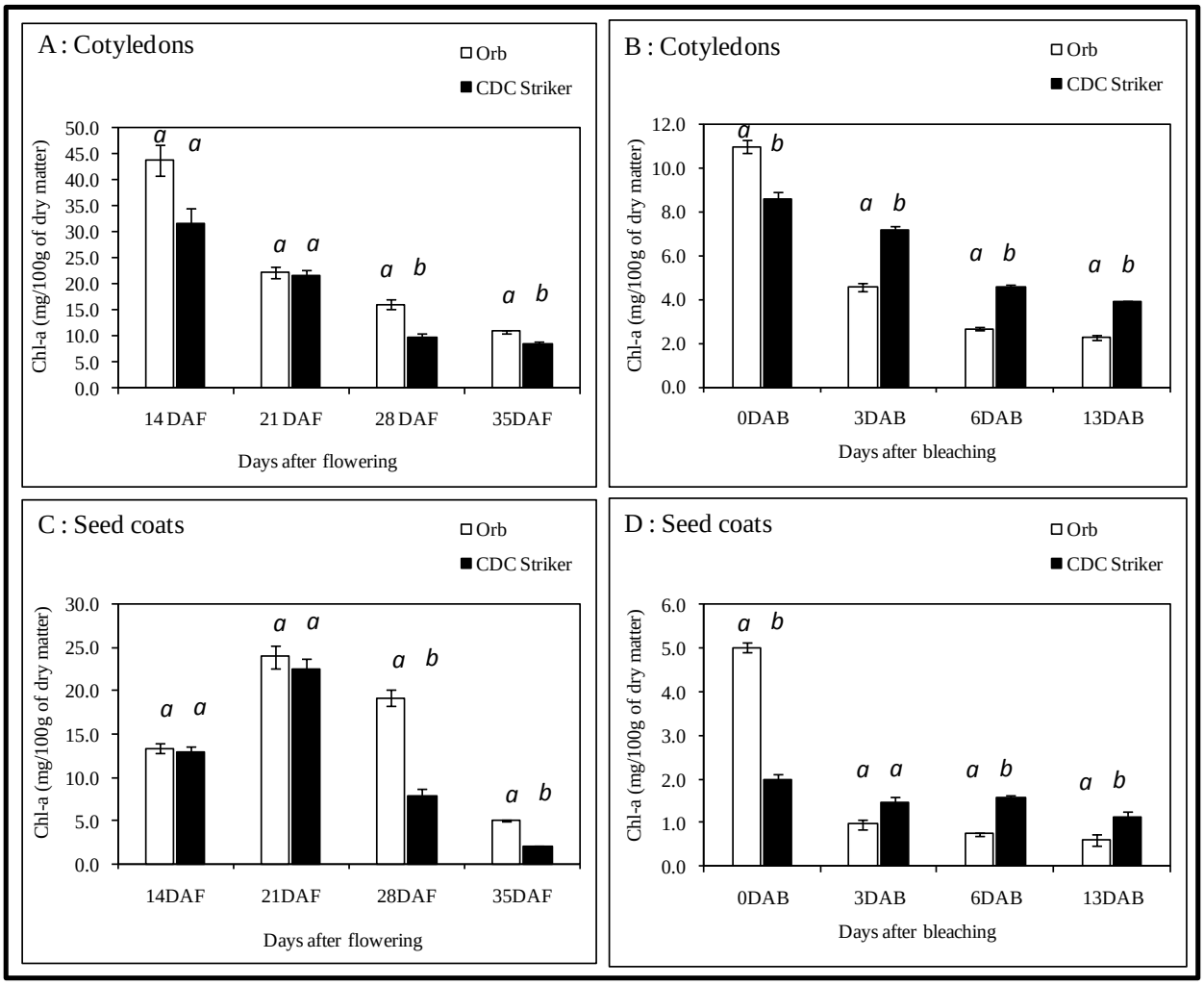

Fig. 5.4. Changes in chlorophyll-a (Chl-a) concentration (mg/100 g) of the cotyledons (A and B) and seed coats (C and D) of two pea cultivars (Orb and CDC Striker) during seed development (A and C) and light mediated bleaching (B and D) periods. Values with the same letter indicated no significant difference based on the LSD at $P \leq 0.05$. Error bars on the graphs indicate ± standard error.

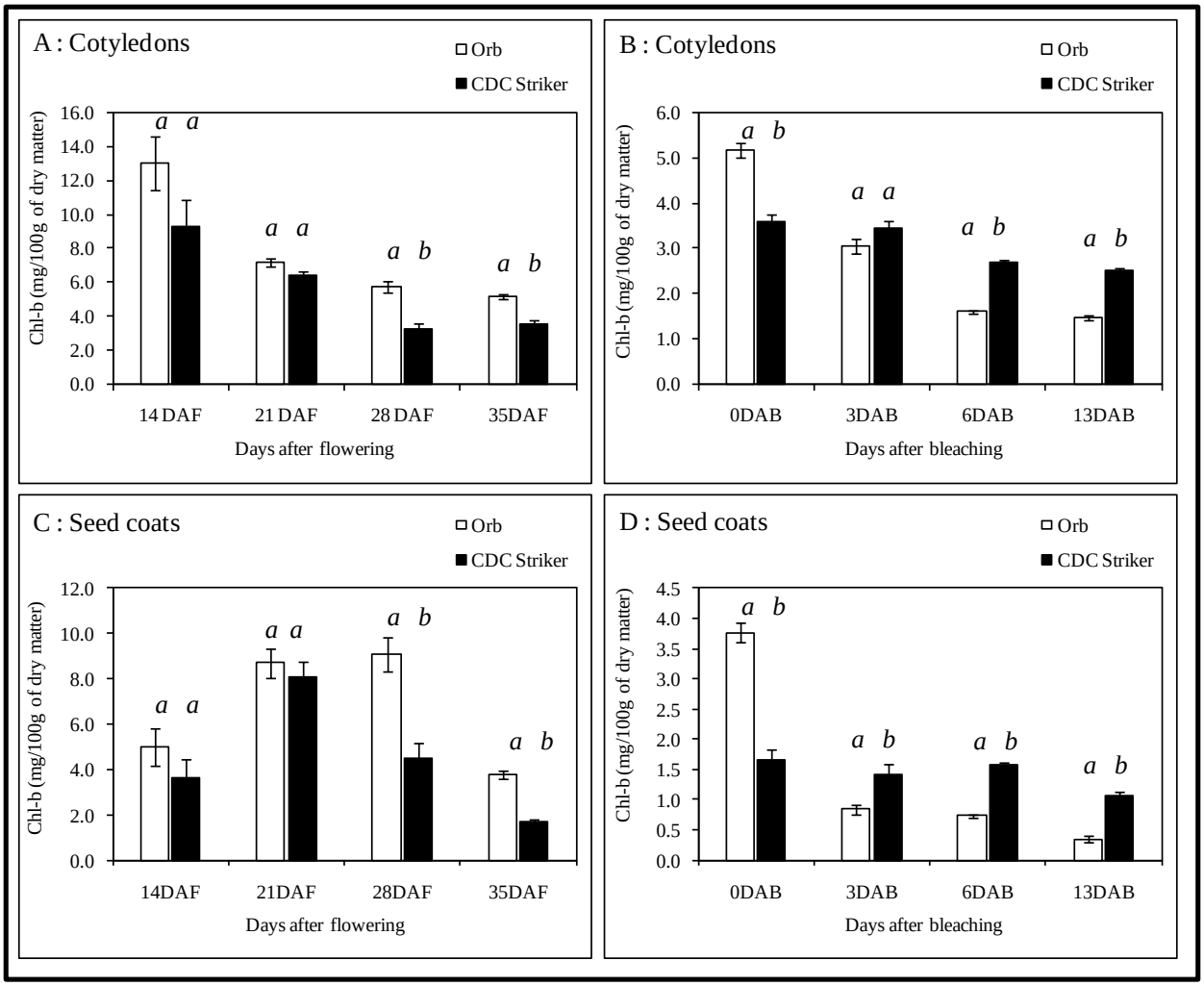

Fig. 5.5. Changes in chlorophyll-b (Chl-b) concentration (mg/100 g) of the cotyledons (A and B) and seed coats (C and D) of two pea cultivars (Orb and CDC Striker) during seed development (A and C) and light mediated bleaching (B and D) periods. Values with the same letter indicate no significant difference based on the LSD at $P \leq 0.05$. Error bars on the graphs indicate ± standard error.

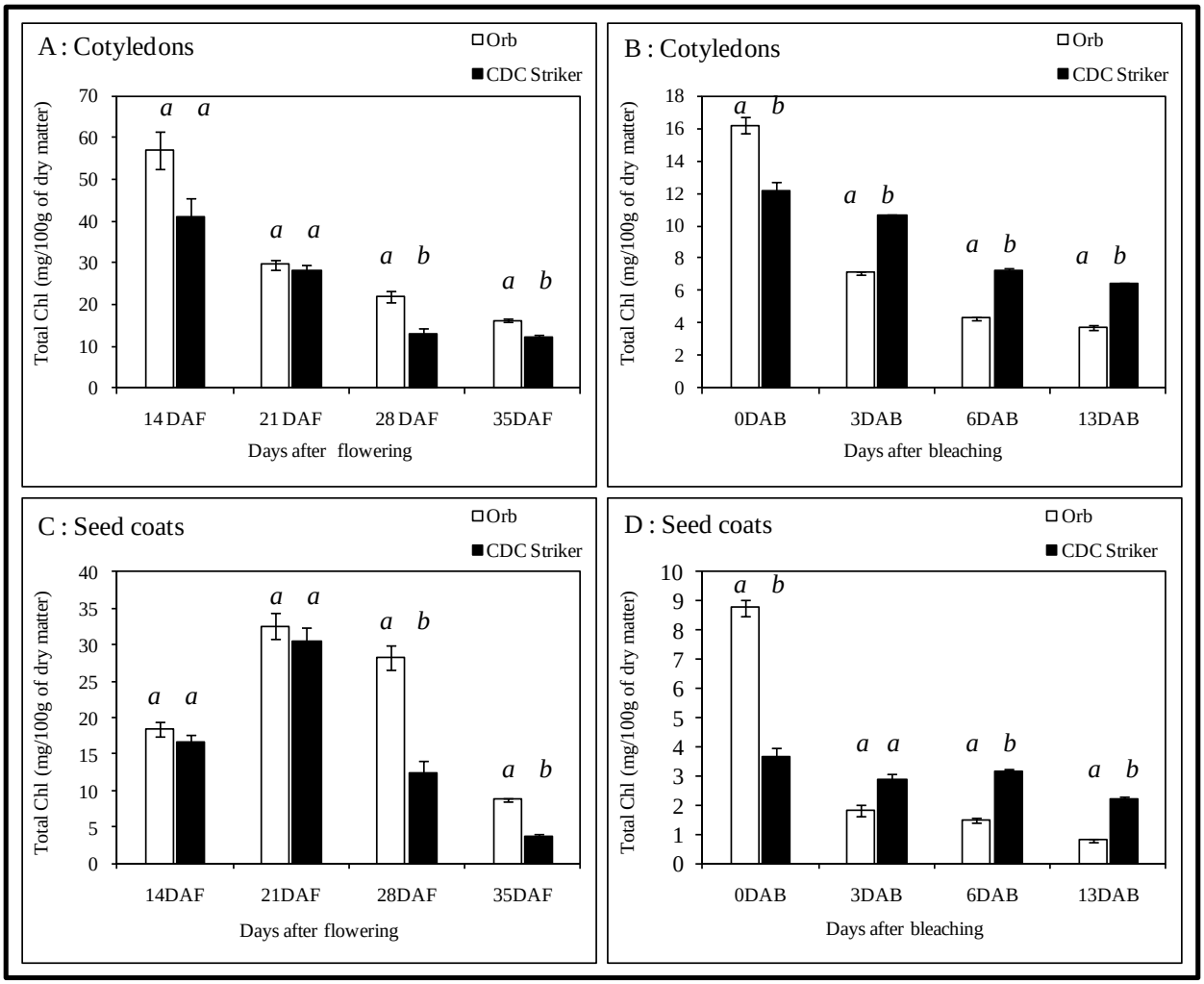

Fig.5. 6. Changes in total chlorophyll concentration (mg/100 g) of the cotyledons (A and B) and seed coats (C and D) of two pea cultivars (Orb and CDC Striker) during seed development (A and C) and light mediated bleaching (B and D) periods. Values with the same letter indicate no significant difference based on the LSD at $P \leq 0.05$. Error bars on the graphs indicate ± standard error.

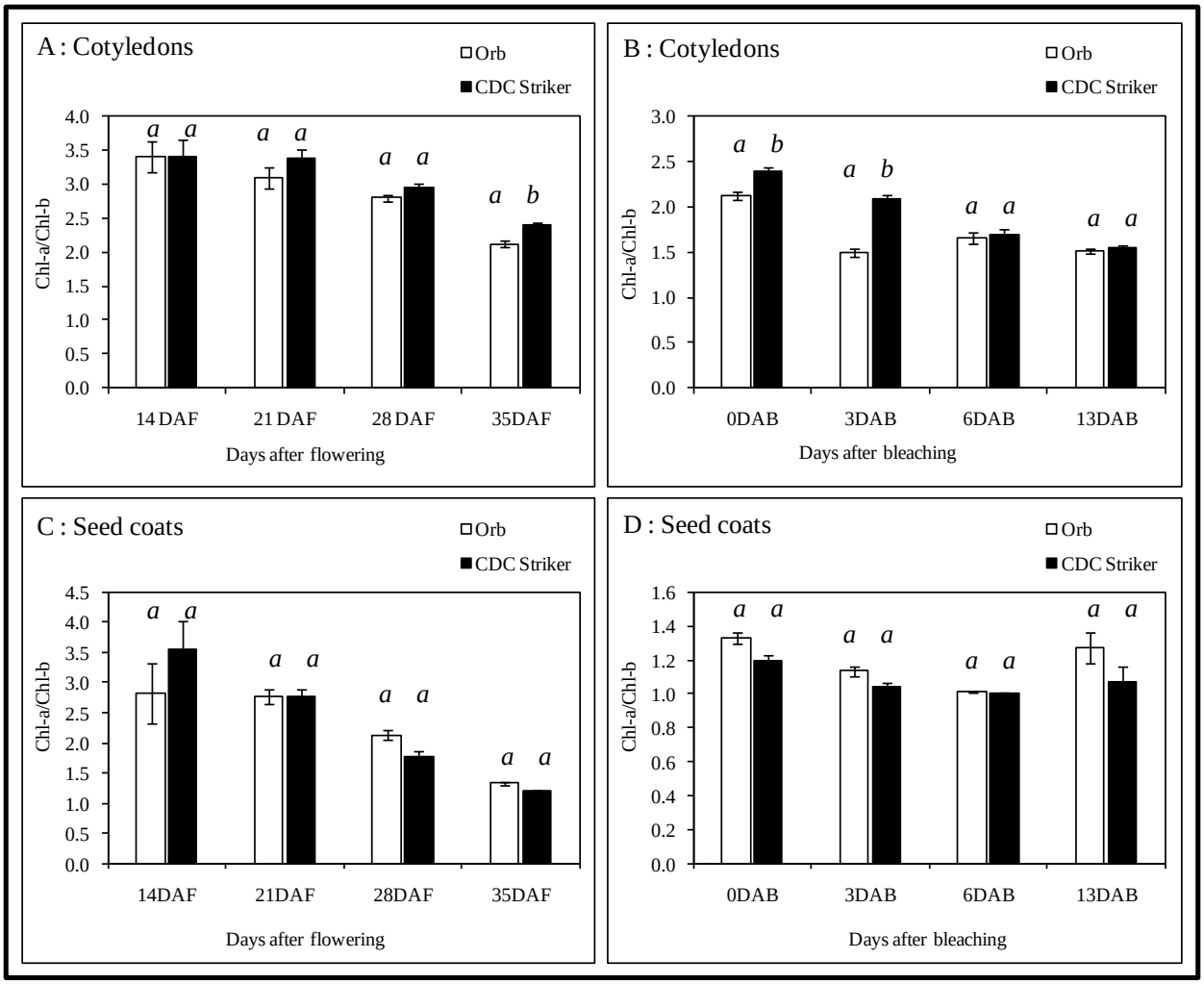

Fig. 5.7. Changes in chlorophyll-a to b ratio of the cotyledons (A and B) and seed coats (C and D) of two pea cultivars (Orb and CDC Striker) during seed development (A and C) and light mediated bleaching (B and D) periods. Values with the same letter indicate no significant difference based on the LSD at $P \leq 0.05$. Error bars on the graphs indicate ± standard error.

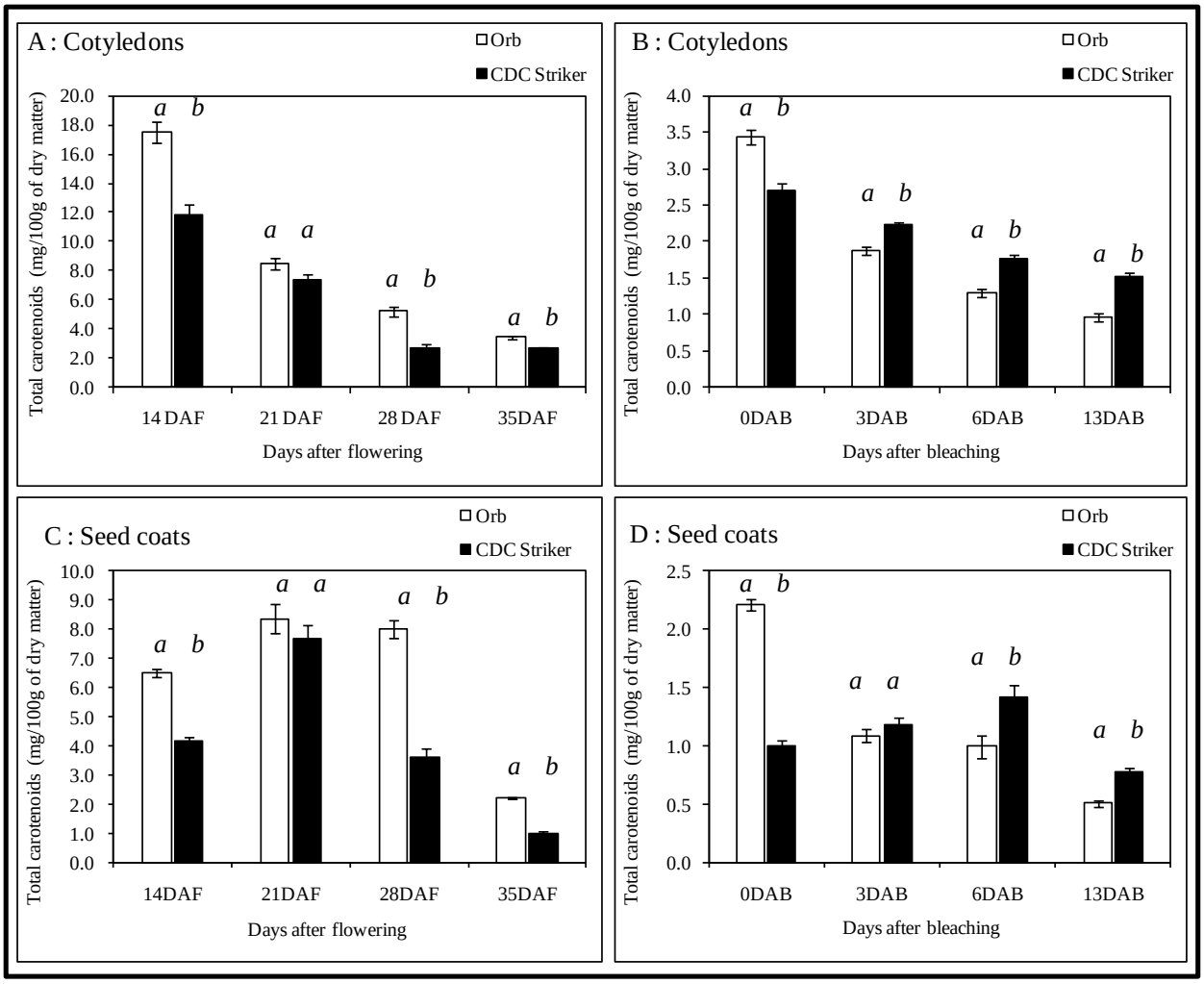

Fig. 5.8. Changes in Total carotenoids concentration (mg/100 g) of the cotyledons (A and B) and seed coats (C and D) of two pea cultivars (Orb and CDC Striker) during seed development (A and C) and light mediated bleaching (B and D) periods. Values with the same letter indicate no significant difference based on the LSD at $P \leq 0.05$. Error bars on the graphs indicate ± standard error.

The trend of chlorophyll pigments (Chl-a, Chl-b and total chlorophyll) from 14 to 35 DAF was linear but no significant difference between these two cultivars were observed. In contrast to the seed developmental stages, the Chl-a, b and total chlorophyll concentrations at different accelerated bleaching stages were significantly different ($P \leq 0.05$) with higher concentration in CDC Striker compared to Orb. These pigment reductions were found to have both linear and quadratic trends with significant differences between cultivars. The ANOVA indicated that the Chl-a to b ratio of the cotyledons between Orb and CDC Striker differed significantly only at 35 DAF and 3 DAB with higher Chl-a to b ratio in CDC Striker cotyledons. The carotenoid concentration of the cotyledons differed significantly between cultivars at all the stages investigated except 21 DAF. Higher concentrations of total carotenoids were observed in Orb cotyledons during the seed developmental periods. However, the total carotenoids concentration was higher in CDC Striker cotyledons during the accelerated bleaching conditions with significantly different linear and quadratic trends between cultivars.

Significant differences in seed coat photosynthetic pigment concentrations were detected between Orb and CDC Striker during seed development and bleaching periods (Table 5.2). The concentration of Chl-a, b and total chlorophylls of the seed coats were significantly higher in Orb at 28 and 35 DAF. However, the concentration of Chl-a, Chl-b and total chlorophylls were higher in CDC Striker during accelerated bleaching with significantly different trends of pigment breakdown between cultivars. No significant differences for the Chl-a/b ratio were observed for both seed development and seed bleaching stages. Significantly higher concentrations of carotenoid in the seed coats of Orb were observed at 28 DAF, 35 DAF and 13 DAB.

A peak at UV absorbance 326 nm wave length was detected with a substantial difference between the seed coat pigment extracts of Orb and CDC Striker (Fig.5.9). Peak differences at 326 nm were also detected between bleached and non-bleached seeds of Orb obtained after exposure to accelerated bleaching (Fig. 5.10). This observation suggested that a chemical difference in seed coats may be associated with bleaching resistance properties. Further characterization of this peak was not conducted in this study.

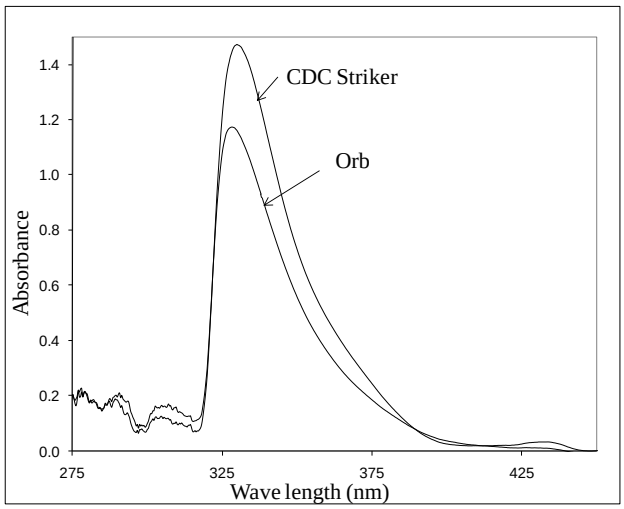

Fig. 5.9. UV absorption spectra of acetone extracts of seed coats of CDC Striker and Orb at harvest (absorption values for 1 mg of sample).

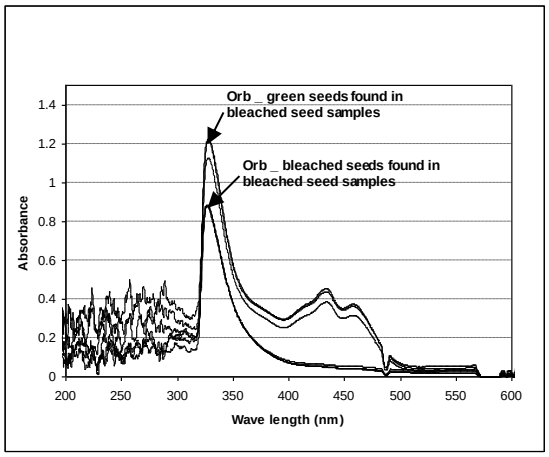

Fig. 5.10. UV absorption spectra of 80% Tris buffered acetone extracts of mature seed coats of Orb after 16 days exposure to light. Note that the same amount of tissue was used in each extract.

However, the association of this peak with the bleaching resistant trait was investigated using a subset of RILs originating from a cross between Orb and CDC Striker (Ubayasena et al. 2010). Table 5.3 summarizes the means and standard errors for the UV absorption at 326 nm of the seed coat extracts along with the phenotypic measurements used to evaluate bleaching resistance (Hunter lab colorimeter "a" value of the whole seeds and cotyledons after exposing seeds to accelerated bleaching conditions). Differences ($P \leq 0.001$) in UV absorbance at 326 nm were detected between the seed coat extracts of 5 bleaching resistant RILs and 5 bleaching susceptible RILs with estimated difference of 2.6 with standard error of 0.2 (Table 5.4). Comparison of the UV absorption at 326 nm and phenotypic measurements (Hunter lab colorimeter "a" value) of the seed and cotyledons indicted significant negative correlations ($P \leq 0.001$) of -0.7 and -0.6, respectively. Thus, increased concentration of compounds in seed coats with UV absorption maxima at 326 nm was associated with greener seeds.

5.4.2 Gene expression profile analysis in developing seed coats

The quality of the intensity measurement detected from each microarray slide was found to be within the acceptable range by observing the intensity ratios of internal replicates and the quality control spots printed on the microarray slides along with the 4946 genes in three replicates (data not shown). Differentially expressed genes of the seed coats of Orb and CDC Striker at three developmental stages (14, 21 and 28 DAF) were recognized as up regulated and down-regulated with the $\log_2$ expression ratio of more than 1.0 ($P \leq 0.05$) (up regulated in CDC Striker or down regulated in Orb) and less than -1.0 ($P \leq 0.05$) (down regulated in CDC Striker or up-regulated in Orb). A total of 184 genes out of 4946 were differentially expressed (up or down regulated) between Orb and CDC Striker seed coats during the seed development stages studied (Fig. 5.11). Of the 184 genes, 72, 30 and 55 genes were differentially expressed at 14, 21 and 28 DAF stages, respectively. A set of genes were recognized as being differentially expressed at more than one seed development stages (18, 2 and 3 genes were identified as differentially expressed at both 14 and 21 DAF, 14 and 28 DAF and 21 and 28 DAF, respectively, while 4 genes were differentially expressed at all three developmental stages).

Table 5.3. Mean UV absorbance at 326 nm for seed coat extracts, and greenness (Hunter Lab colorimeter "a" values') of bleached seeds (BWSa) and cotyledons (BDSa) of parental cultivars and selected RILs grown at two locations over two years.

Cultivars and RILs tested		Phenotypic measurement of bleaching		UV Absorbance at
		BWSa[†]	BDSa[#]	326nm*
Bleaching resistant	CDC Striker	-1.5±0.2	-3.8±0.4	1.3±0.1
	RIL1-08	-1.9±0.2	-4.6±0.4	1.5±0.1
	RIL1-13	-1.9±0.2	-4.3±0.4	1.6±0.1
	RIL1-50	-2.0±0.2	-4.4±0.4	1.8±0.1
	RIL1-16	-1.5±0.2	-4.2±0.4	1.5±0.1
	RIL1-65	-1.9±0.2	-4.1±0.4	1.6±0.1
Bleaching susceptible	Orb	0.5±0.2	-0.3±0.4	1.0±0.1
	RIL1-21	-0. 7±0.2	-0.8±0.4	1.3±0.1
	RIL1-48	0.1±0.2	-0.8±0.4	1.2±0.1
	RIL1-35	-0.1±0.2	-0.8±0.4	0.8±0.1
	RIL1-69	0.3±0.2	-0.6±0.4	0.9±0.1
	RIL1-54	-0.2±0.2	-1.4±0.4	1.1±0.1

Note: [†], Hunter Lab colorimeter "a" value of bleached whole seeds (BWSa)

[#], Hunter Lab colorimeter "a" value of bleached cotyledons (BDSa)

*, 1 g of ground seed coat was used in the extraction with 80% Tris buffered acetone.

Table 5.4. Partial analysis of variance with mean squares and significance levels for the UV absorbance at 326 nm for the seed coat extracts, greenness of bleached seeds and cotyledons estimated by Hunter Lab colorimeter "a" values for the parental and selected RILs with extreme reaction to bleaching resistance grown at two locations over two years.

Source	df	UV326[§]		BWSa [†]		BDSa[#]	
		Estimate±Std E	F value	Estimate±Std E	F value	Estimate±Std E	F-value
Entry[¥]	11	-	20.2***	-	27.3***	-	20.9***
CDC Striker – Orb ¶	1	0.3±0.1	8.5*	-2.0±0.3	55.2***	-3.5±0.6	39.7***
Resistant RILs - Susceptible RILs¶	1	2.6±0.2	55.2***	-9.3±0.6	226.9***	-17.1±1.3	182.8***

Note: [¥], two cultivars and selected RILs of Orb/CDC Striker population, ¶, analyzed contrasts, §, UV absorbtion at 326nm wave length of seed coat extract, [†], Hunter Lab colorimeter "a" value of bleached whole seeds (BWSa), [#], Hunter Lab colorimeter "a" value of bleached cotyledons (BDSa), [*], significant at $P \leq 0.05$, [**], significant at $P \leq 0.01$, [***], Significant at $P \leq 0.001$.

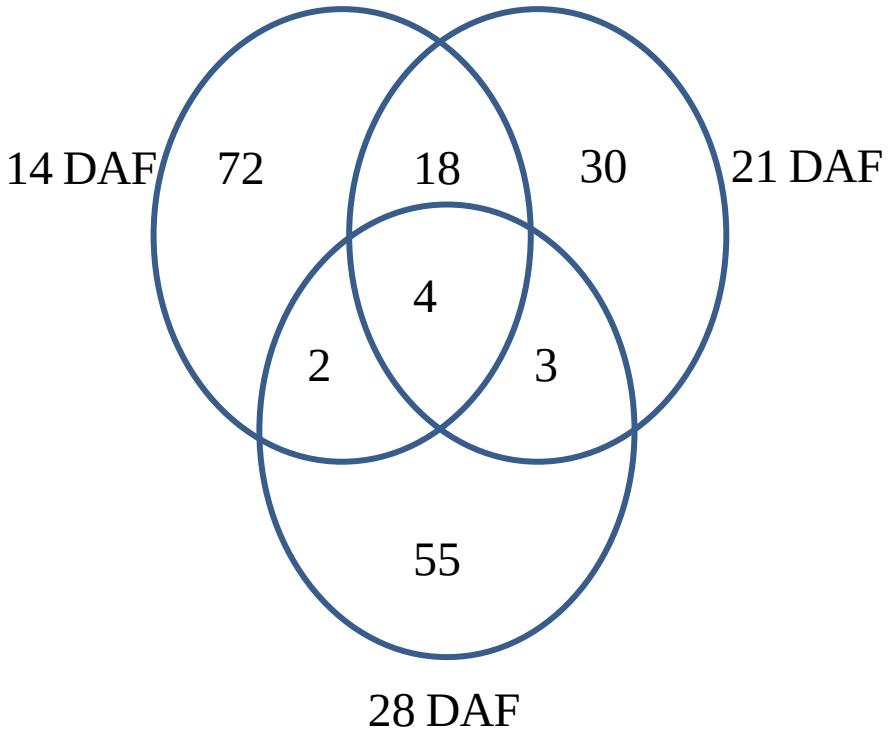

Fig. 5.11. Venn diagram for the number of genes differentially expressed between Orb and CDC Striker seed coats at three developmental stages. Genes were considered as differentially expressed if the Log_2 expression ratios were between 1.0 and -1.0 ($P \leq 0.001$).

The complete list of genes differentially expressed at 14, 21 and 28 DAF with their recent annotation and functional groups are given in Appendix 7, 8 and 9, respectively. A total of 47, 28 and 20 up regulated genes and 49, 26 and 44 down regulated genes were identified in CDC Striker seed coats compared to Orb at 14, 21 and 28 DAF stages, respectively. Higher numbers of differentially expressed genes in seed coats at early and late stages of seed development in both cultivars were observed compared to the middle stage (21 DAF).

Of these differentially expressed genes, 15 (14 DAF), 8 (21 DAF) and 6 (28 DAF), had no homology to previously annotated genes. The previously annotated genes were classified into six groups based on their involvement in cellular functions including maturation growth and development, metabolism, stress and pathogen response, transcription and translation, transport and protein processing, and signal transduction (Appendix 7, 8, 9 and Fig. 5.12). Many genes associated with metabolism, maturity growth and development, and transport and protein processing were differentially expressed at 14 DAF compared to the other two stages. Thus, differential genetic regulation of seed coat functions occurred during late embryogenesis and the onset of grain filling.

The majority of the genes up regulated in the seed coats of Orb at 14, 21 and 28 DAF were recognized as transcripts involved in photosynthesis, chlorophyll metabolism, chloroplast assembly, carbohydrate metabolism and amino acid metabolism. A major portion of up regulated transcripts in seed coats of CDC Striker at these three developmental stages indicated proteins and enzymes involved in membrane integrity, programmed cell proliferation and death, photoprotection of chloroplast, stress signaling and cell redox reactions. Most of the genes up regulated in the seed coats of Orb at all three developmental stages compared to CDC Striker were related to metabolic activity. The beginning of seed maturity and desiccation were characterized by reduced gene activity, mainly signal transduction and transport and protein accumulation at 28 DAF stages of both cultivars except the genes involved in metabolic functions of both cultivars and stress and pathogen related in Orb.

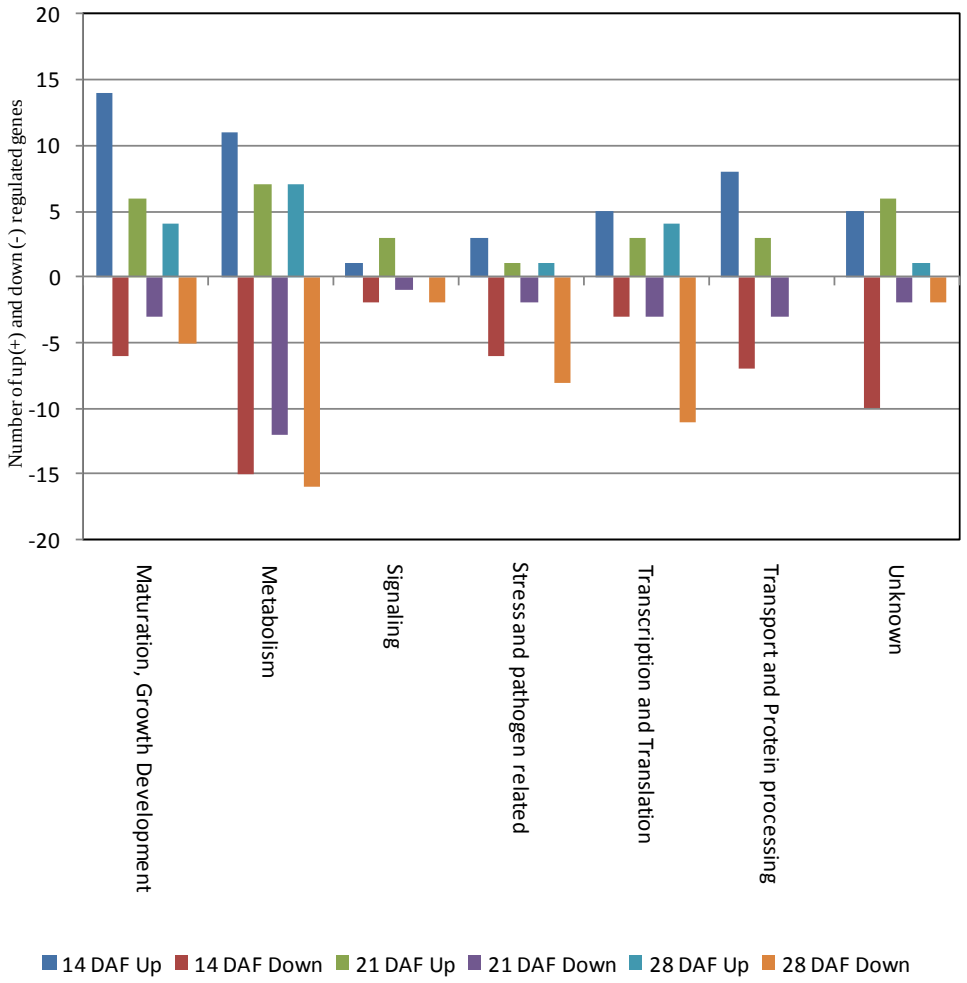

Fig. 5.12. Number of genes up- or down regulated in different functional groups in CDC Striker compared to Orb seed coats at three developmental stages. For the details of the genes refer to Appendix 7, 8 and 9.

5.4.3 Global pattern of seed coat gene expression between CDC Striker and Orb

Hierarchical clustering of mean $\log_2$ expression ratios was performed to observe the global pattern of differential gene expression between CDC Striker and Orb at three developmental stages (Fig 5.13). Twenty-seven clusters indicated by different branch color in Fig. 5.13 were identified. Of the 27 clusters, 11 were selected based on the median $\log_2$ expression value, ≥ 1 or ≤ -1, of the genes at any developmental stage (Fig 5.14). The number of genes in each cluster ranged from 5 (cluster C) to 21 (cluster B) (Appendix 10).

Cluster A mainly consisted of genes significantly up regulated in Orb compared to CDC Striker at 28 DAF. High levels of transcript abundance for the genes responsible for photosynthesis were identified. Two genes, PSOLI04570 and PSOLI05071, responsible for cellular protection during seed maturation were up regulated indicating early stage of transition from grain filling to desiccation and maturation. Among the stress related genes, PSOLI04798 and PSOLI04608 were up regulated in Orb seed coats.

The majority of the genes grouped into cluster B and C were up regulated at 14 and 21 DAF in the seed coats of CDC Striker compared to Orb. These genes were significantly up regulated in early and mid (14 and 21 DAF) seed developmental stages in CDC Striker indicating a high level of cellular activity related to cell division, differentiation and diverting metabolites from the seed coats toward the growing embryo. In addition, two genes responsible for the proteins involved in maintaining redox potential of the cells, (PSOLIO104890 and PSOLIO4233) were significantly up regulated at 14 and 21 DAF stages of the CDC Striker seed coats.

The five genes grouped into cluster D include one gene each controlling metabolism, signaling, transcription and translation, and transport and protein processing.The genes within this cluster had peak up regulation at 21 DAF in CDC Striker seed coats. Cluster E contains 13 up regulated genes at 14 DAF in CDC Striker seed coats including transcripts associated with maturation, growth and development and transport and protein processing. In addition, two genes responsible for amino acid metabolism, one gene each for stress and pathogen resistance and transport and translation were grouped together with the same pattern of expression.

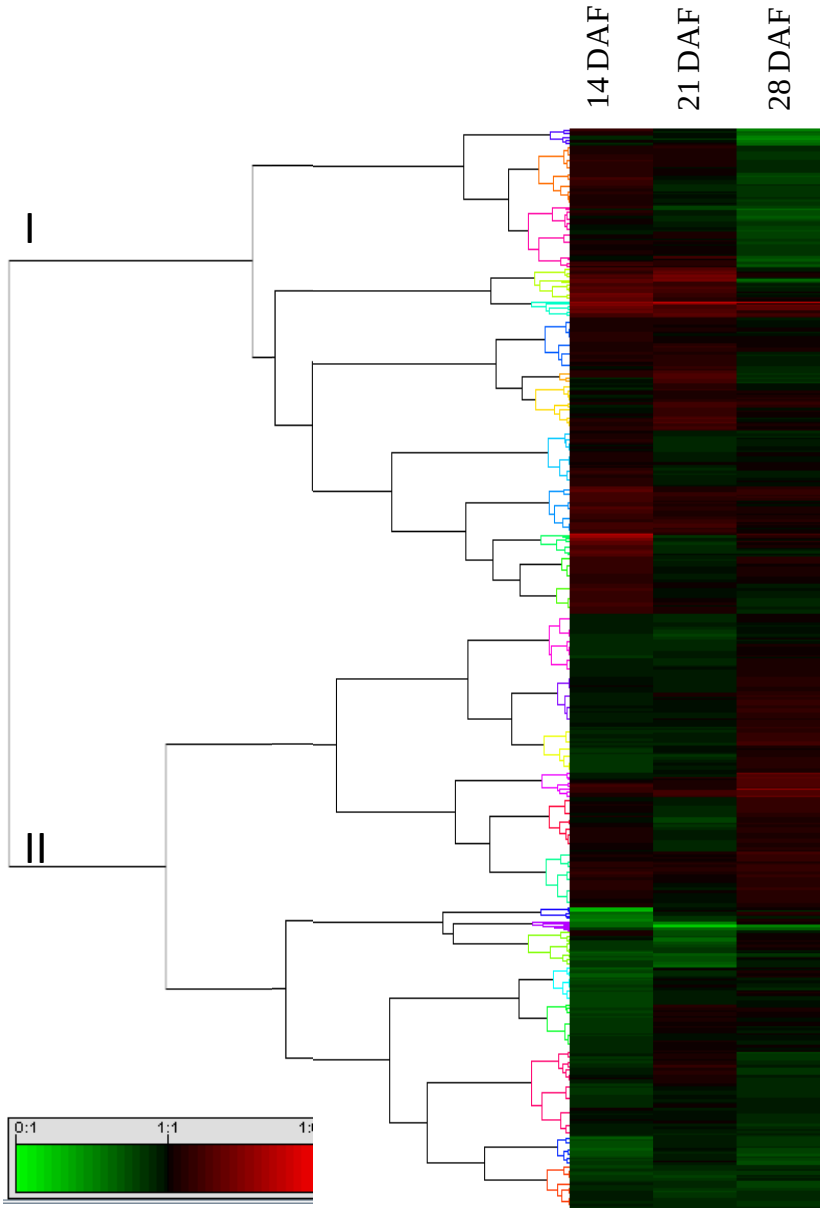

Fig. 5.13. Hierarchical clustering of the genes with significant differential expression profiles between two cultivars (CDC Striker and Orb) across three developmental stages (14, 21 and 28 DAF) in seed coats. The red bars indicate the up regulated genes in Orb (down regulated in CDC Striker) and the green bars indicate the genes down-regulated in Orb (up regulated in CDC Striker).

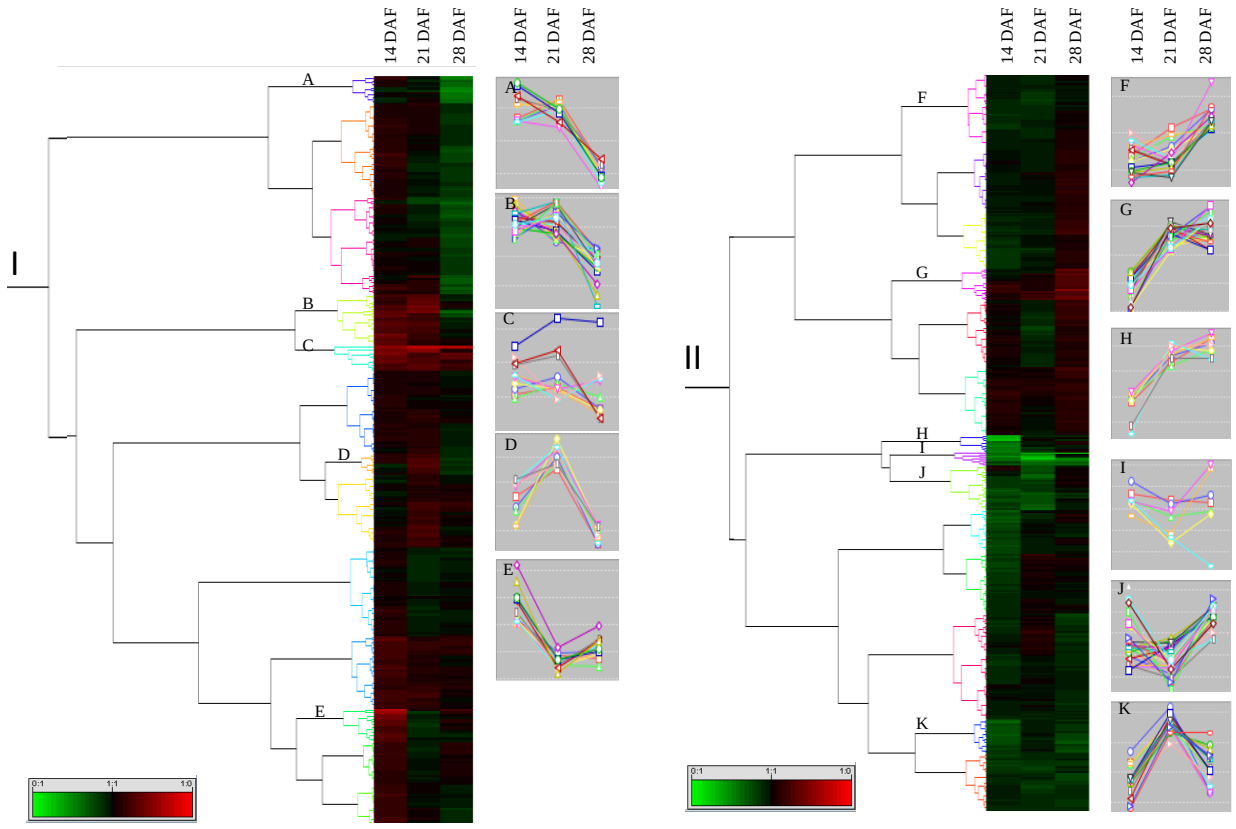

Fig. 5.14. A detailed view of the hierarchical clustering of the genes with significant differential expression profiles between two cultivars (CDC Striker and Orb) across three developmental stages (14, 21 and 28 DAF) in seed coats. The red bars indicate the up regulated genes in Orb (down regulated in CDC Striker) and the green bars indicate the genes down-regulated in Orb (up regulated in CDC Striker). Clusters labeled with letters A to K represent the different groups identified based on the median gene expression value of the expression ratio at different developmental stages. The panels on the right illustrate the transcription pattern of the identified gene clusters.

Most of the genes grouped into cluster F, were up regulated at 28 DAF in CDC Striker seed coats except PSOLIO1214. These genes are mainly responsible for the production of membrane protein associated with cell wall integrity and adhesion. The majority of the genes in this cluster were associated with metabolism including amino acid metabolism, lipid metabolism, secondary metabolites metabolism, photosynthesis and respiration.

The genes grouped into clusters G, H and K were identified as mainly up regulated in Orb seed coats at 14 DAF. The genes in clusters G and H were responsible for functions related to the early stages of seed coat development including cell division, differentiation, and metabolite transport to the growing embryo. Six of the 15 genes grouped into cluster K had unknown functions.

Cluster I consisted of genes up regulated at all three developmental stages in Orb seed coats. Thus, these genes might be necessary during the whole period of seed development to regulate the activities of seed coat related functions. This group contained two genes related to metabolism, PSOLI10981 and PSOLI00894. These are associated with the regulation of amino acid glycosylation, chloroplast development, maturation growth and development, signaling, transcription and translation and transport and protein processing.

5.4.4 KEGG pathway analysis of differentially expressed genes in biosynthesis pathways

Based on the genetics and biochemical studies conducted on green cotyledon bleaching resistance in pea in this study, several important biosynthetic pathways leading to the production of chlorophyll, carotenoids and several secondary metabolites are given in Fig. 5.15-5.19. For an individual gene, histograms with three bars indicate transcriptional up and down regulation at 14, 21 and 28 days, respectively, from left to right. Green colored histograms indicate a significant up regulation ($P \leq 0.05$) of transcripts in CDC Striker seed coats compared to Orb seed coats at that stage, while red color indicates down regulation. Current annotations of the genes involved in these pathways are given in Table 5.5.

The genes tagged by the KEGG pathway analysis of porphyrin and chlorophyll metabolism indicate that several genes are responsible for the biosynthesis of Chl-a and Chl-b, and these genes appear to be up-regulated in Orb seed coats compared to CDC Striker (Fig. 5.15 and Table 5.5).

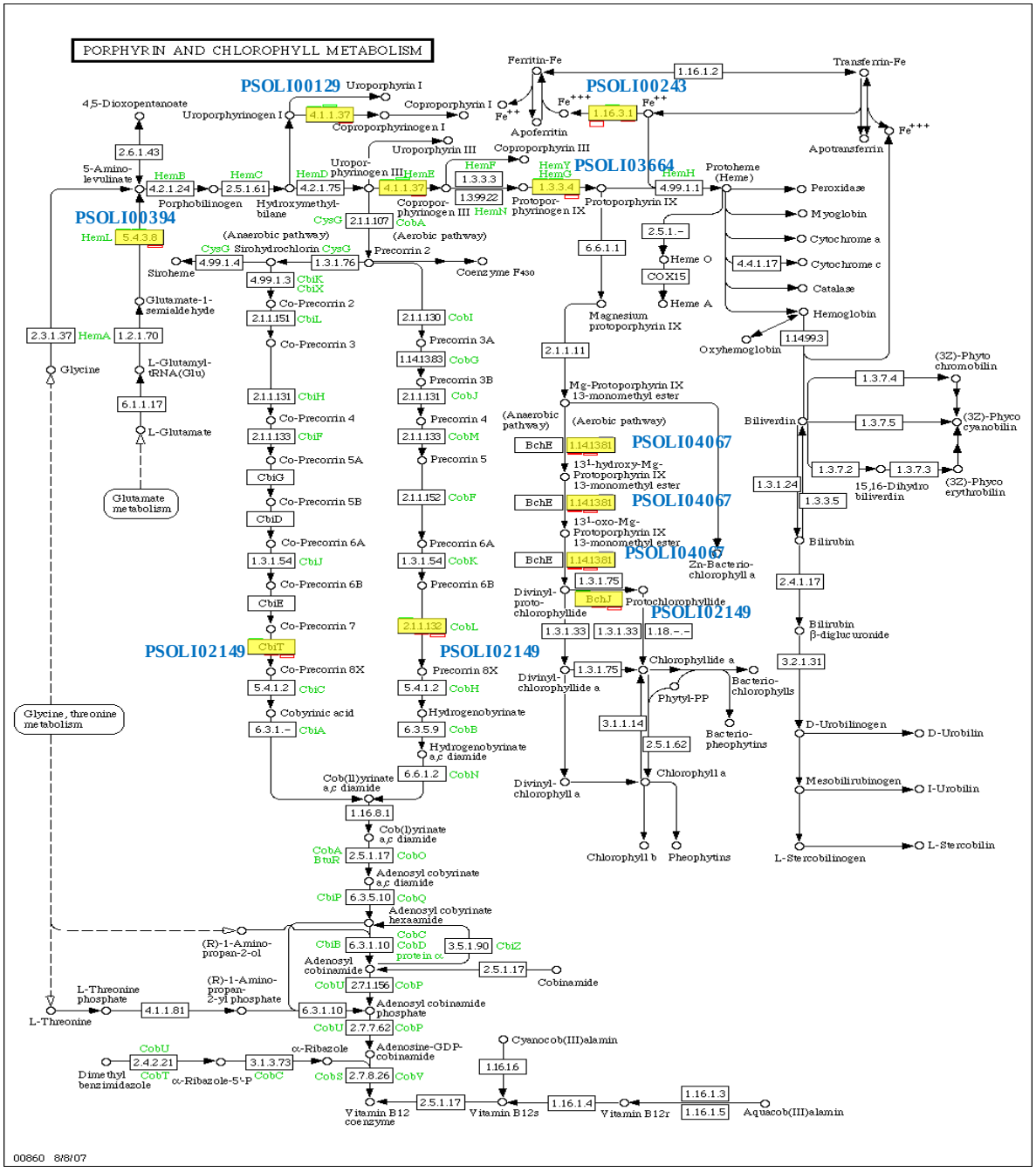

Fig. 5.15. Differential gene expression pattern within the porphyrin and chlorophyll biosynthesis pathway of seed coats of CDC Striker and Orb at 14, 21 and 28 DAF. Yellow boxes indicate that the gene expression responsible for a particular step is significantly different ($P \leq 0.05$) between cultivars and the responsible gene ID is given as PSOLI number. Refer to Table 5.5 for gene annotation. Green (CDC Striker up regulated) and red (Orb up regulated) histograms represent the transcript profiling at 14, 21 and 28 DAF.

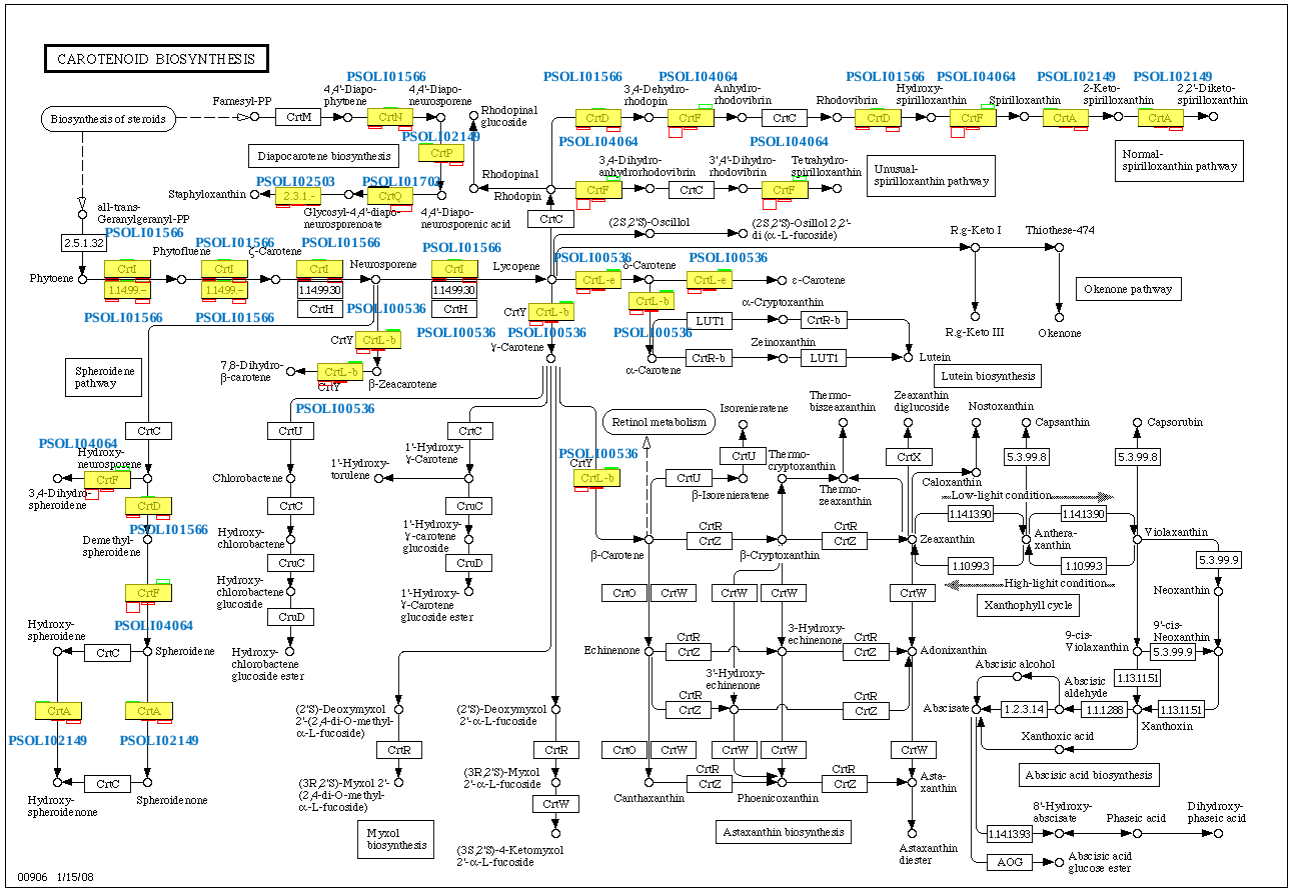

Fig. 5.16. Differential gene expression pattern within the carotenoid biosynthesis pathway of the seed coats of CDC Striker and Orb at 14, 21 and 28 DAF. Yellow boxes indicate that the gene expression responsible for a particular step is significantly different ($P \leq 0.05$) between cultivars and the responsible gene ID is given as PSOLI number. Refer to Table 5.5 for gene annotation. Green (CDC Striker up regulated) and red (Orb up regulated) histograms represent the transcript profiling at 14, 21 and 28 DAF.

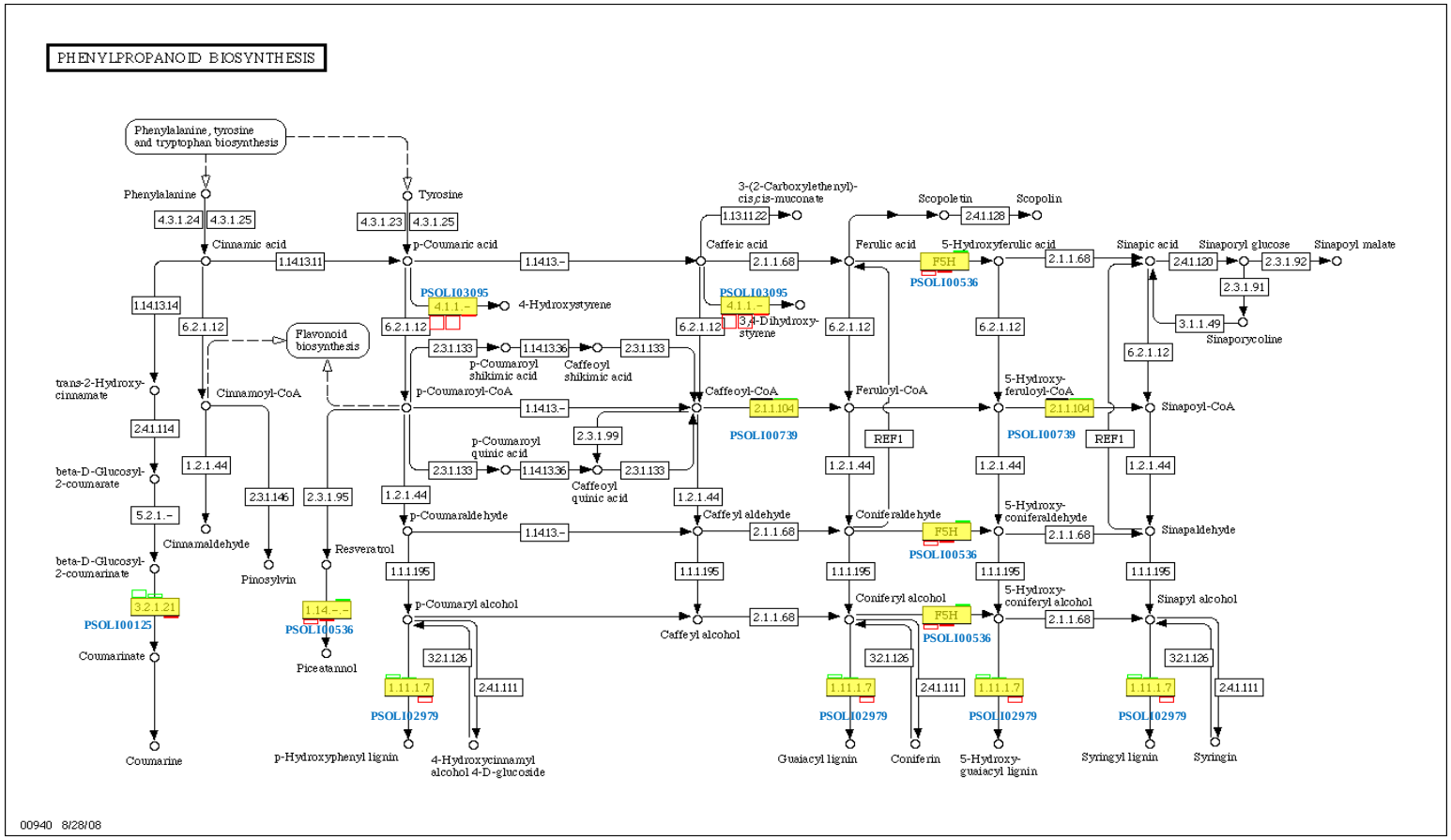

Fig. 5.17. Differential gene expression pattern within the phenylpropanoid biosynthesis pathway of seed coats of CDC Striker and Orb at 14, 21 and 28 DAF. Yellow boxes indicate that the gene expression responsible for a particular step is significantly different ($P \leq 0.05$) between cultivars and the responsible gene ID is given as PSOLI number. Refer to Table 5.5 for gene annotation. Green (CDC Striker up regulated) and red (Orb up regulated) histograms represent the transcript profiling at 14, 21 and 28 DAF.

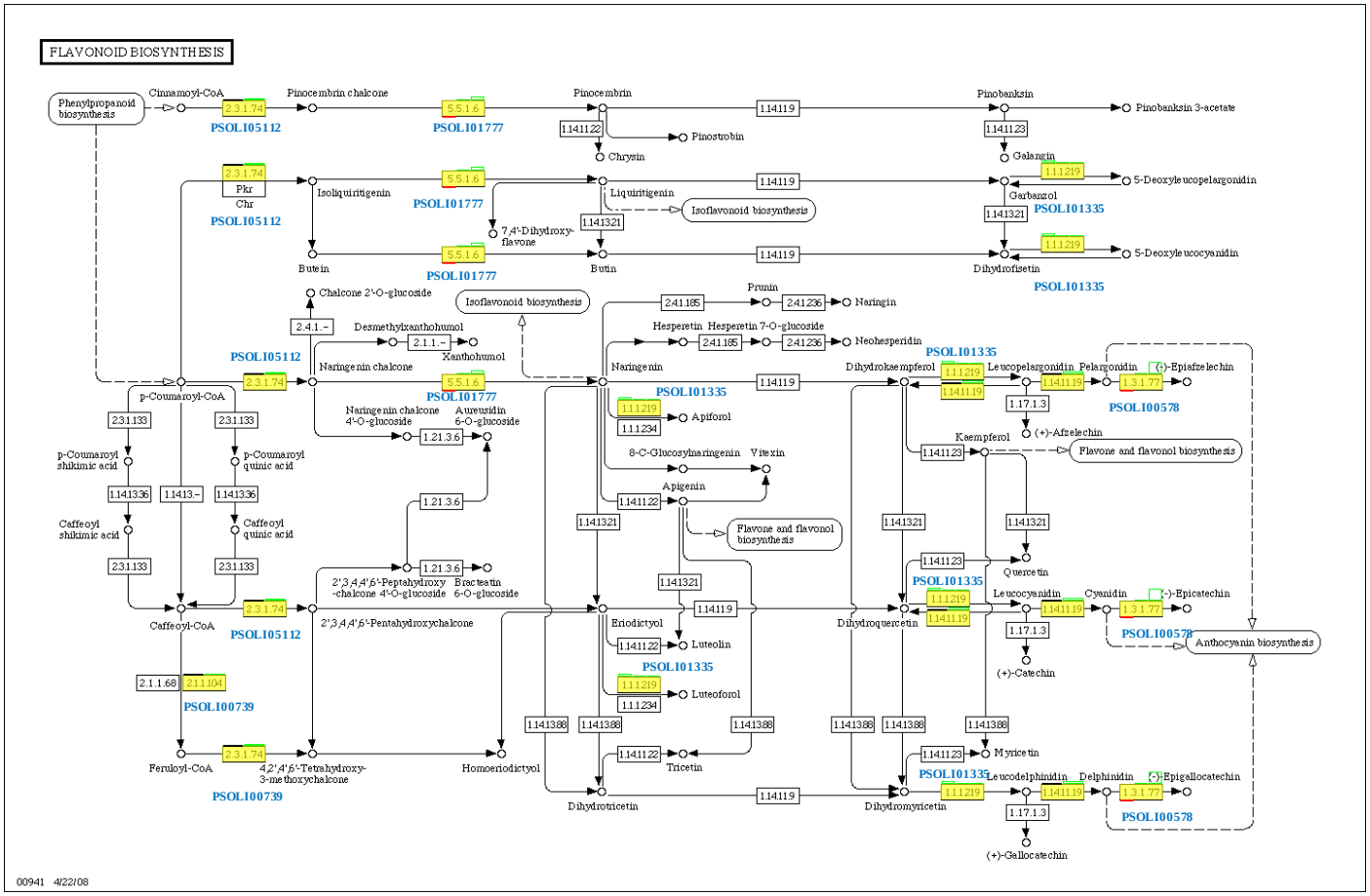

Fig. 5.18. Differential gene expression pattern within the flavonoid biosynthesis pathway of seed coats of CDC Striker and Orb at 14, 21 and 28 DAF. Yellow boxes indicate that the gene expression responsible for a particular step is significantly different ($P \leq 0.05$) between cultivars and the responsible gene ID is given as PSOLI number. Refer to Table 5.5 for gene annotation. Green (CDC Striker up regulated) and red (Orb up regulated) histograms represent the transcript profiling at 14, 21 and 28 DAF.

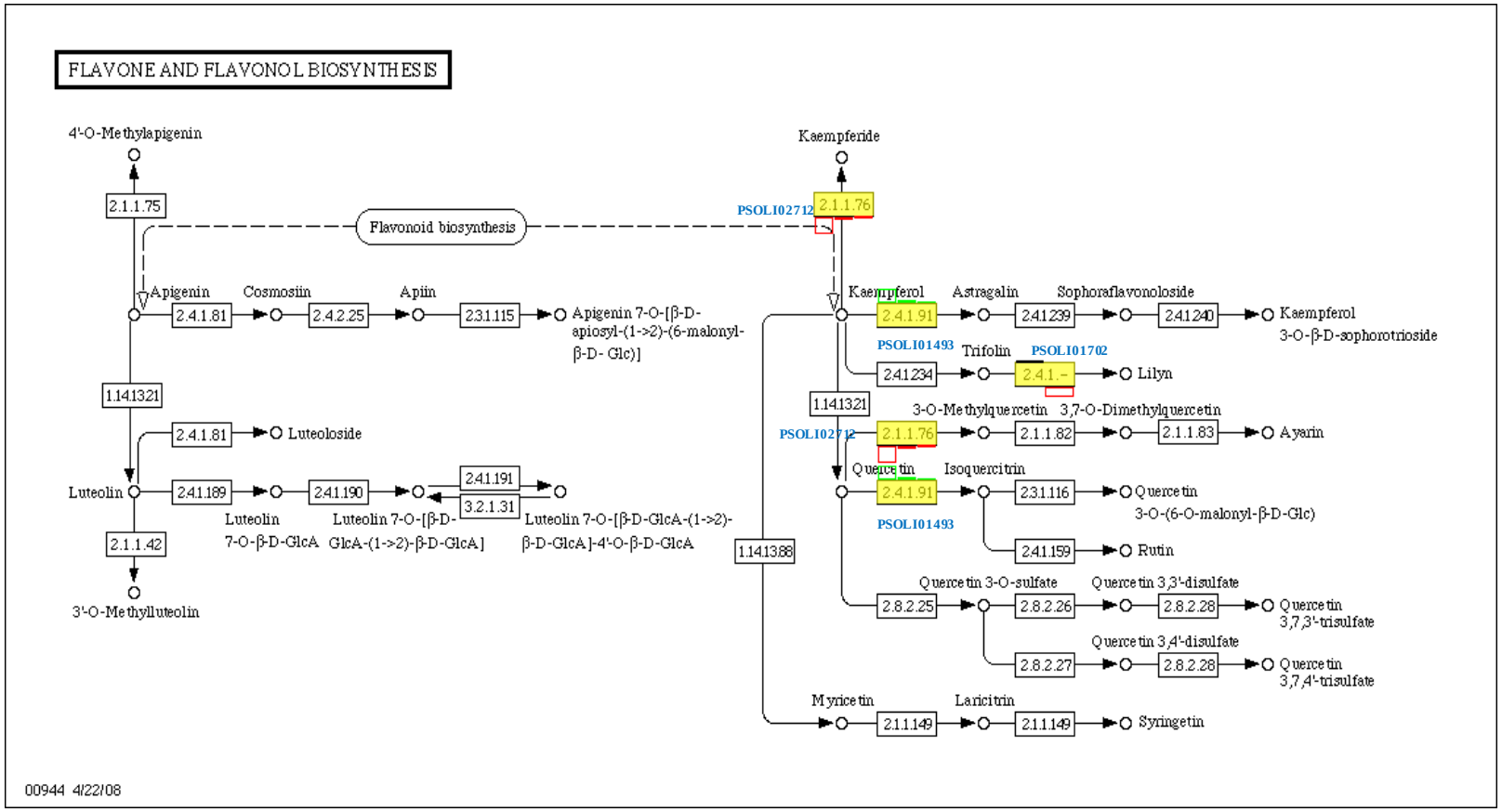

Fig. 5.19. Differential gene expression pattern within the flavone and flavonol biosynthesis pathway of seed coats of CDC Striker and Orb at 14, 21 and 28 DAF. Yellow boxes indicate that the gene expression responsible for a particular step is significantly different ($P \leq 0.05$) between cultivars and the responsible gene ID is given as PSOLI number. Refer to Table 5.5 for gene annotation. Green (CDC Striker up regulated) and red (Orb up regulated) histograms represent the transcript profiling at 14, 21 and 28 DAF.

Table 5.5. List of genes differentially expressed in secondary metabolites, chlorophyll and carotenoids biosynthesis pathway based on the KEGG pathway analysis

Gene ID	Gene annotation	Expression ration (M*)		
		14 DAF	21 DAF	28 DAF
Porphyrin and chlorophyll metabolism				
PSOLI00243	Ferritin 3	-0.71	0.00	-0.14
PSOLI04067	Leucine zipper-containing protein	-0.21	-0.33	0.00
PSOLI02149	Hypothetical protein AT4g37000	0.09	-0.17	-0.46
PSOLI02149	Hypothetical protein AT4g37000	0.09	-0.17	-0.46
PSOLI00129	Uroporphyrinogen decarboxylase	0.01	0.28	-0.13
PSOLI00394	Glutamate-1-semialdehyde 2,1-aminomutase	0.03	0.00	-0.32
PSOLI03664	Protoporphyrinogen oxidase	0.02	0.00	-0.53
Carotenoid biosynthesis				
PSOLI00536	Ferulate 5-hydroxylase	-0.57	-0.26	0.12
PSOLI02503	Fiddlehead-like protein	-0.37	-0.04	-0.15
PSOLI04064	Putative fibrillarin	-1.18	-0.30	0.45
PSOLI01566	Omega-3 fatty acid desaturase	-0.33	0.02	-0.56
PSOLI02149	Chlorophyll catabolite reductase	0.08	-0.17	-0.46
PSOLI01702	Cytokinin-N-glucosyltransferase 1	0.00	0.15	-0.77
Phenylpropanoid biosynthesis				
PSOLI03095	Phosphonopyruvate decarboxylase-like protein	-1.43	-1.46	-0.05
PSOLI00536	Ferulate 5-hydroxylase	-0.56	-0.26	0.12
PSOLI02979	Peroxidase	0.38	0.09	-0.66
PSOLI00125	Non-cyanogenic beta-glucosidase	0.79	0.37	-0.27
PSOLI00739	Caffeoyl-CoA O-methyltransferase	0.00	0.94	0.00
Flavonoid biosynthesis				
PSOLI00578	Hydroxydihydrodaidzein reductase	-0.01	0.00	1.28
PSOLI01777	Putative chalcone isomerase	-0.02	0.03	0.36
PSOLI05112	Naringenin-chalcone synthase	0.00	0.00	0.14
PSOLI00739	caffeoyl-CoA O-methyltransferase	0.00	0.94	0.00
PSOLI01335	SulA protein precursor	0.21	0.01	0.00
Flavone and Flavonol biosynthesis				
PSOLI02712	Caffeic acid 3-O-methyltransferase	-1.29	-0.23	-0.06
PSOLI01493	UDP-glucose glucosyltransferase	0.95	0.13	0.01
PSOLI01702	Glucosyl transferase, putative	0.00	0.14	-0.76

M*: The mean centered relative gene expression value (in $\log_2$ scale)

Among them a leucine zipper-containing protein group (PSOLI04067) and a hypothetical protein identified from *Arabidopsis* AT4g3700 (PSOLI021149) were involved directly in the Chl-a and Chl-b biosynthesis. Several other genes supplying the precursor molecules for Chl-a and Chl-b biosynthesis and ferrous storage were up-regulated in Orb at 28 DAF compared to CDC Striker seed coats. These results support the biochemical profiles reported in this study whereby Orb seed coats had higher concentration of Chl-a and Chl-b during seed development.

The KEGG pathway analysis of carotenoids biosynthesis is given in Figure 5.16 and Table 5.5. Six genes involved in the carotenoid biosynthesis pathway showed differential regulation between cultivars at different developmental stages. Most of the genes show a higher level of transcription in Orb seed coats at 14, 21and 28 DAF. Putative fibrillarin family gene (PSOLI04064) involved in the synthesis of an important group of carotenoids, 3,4 Dihydrospheroidene, spheroidene and spirilloxanthin, were up-regulated in Orb seed coats at 14 DAF and in CDC Striker seed coats at 28 DAF.

Figure 5.17 illustrates the phenylpropanoid biosynthesis pathway with genes included in the Ps6kOLI1 cDNA microarray. This pathway indicated that the gene, phosphonopyruvate decarboxylase-like protein (PSOLI03095), responsible for the conversion of p-Coumaric acid and caffeic acid to hydroxystyrene and 3,4 Dihydroxystyrene, respectively, were significantly up-regulated in Orb. This suggests that the conversion of p-Coumaric acid to p-Coumaroyl CoA, the precursor for the flavanoid biosynthesis pathway, is more efficient in CDC Striker; therefore, the net synthesis of p-Coumaroyl CoA should be higher in CDC Striker seed coats at all three developmental stages compared to Orb. The transcriptional differences observed within the flavanoid biosynthesis pathway are given in Fig. 5.18 and Table 5.5. Most of the genes identified by the KEGG pathway analysis from these microarray results of the flavanoid pathway indicated up-regulation of the transcript level in developing seed coats of CDC Striker (Table 5.5). Increased transcript level of hydroxydihydrodaidzein reductase (PSOLI00578) was observed at 28 DAF in CDC Striker seed coats. This family of reductases is involved in the synthesis of a series of secondary metabolites which are responsible for antioxidant properties in plants tissues such as epiafzelechin, epcatechin and epigallocatechin.

Another important biosynthesis pathway producing secondary metabolites with antioxidant properties in plants tissues is the flavone and flavonol biosynthesis pathway. This pathway is linked with the flavanoid pathway through kaempferol (Fig. 5.19). Three gene

families were identified with significant differential regulation between cultivars at three developmental stages.

Significant up-regulation of caffeic acid 3-O-methyltransferase (PSOLI02712) responsible for the conversion of kaempferol to kaempferide and quercetin to 3-O-methylquercetin were up-regulated in all three stages of seed development in Orb seed coats. In CDC Striker seed coats, high transcript level of the gene UDP-glucose glucosyltransferase (PSOLI01493) was observed. This gene is responsible for the conversion of kaemferol to astragalin and quercetin to isoquercetin. This suggests that the synthesis of kaempferol 3-O-β-D-sophorotrioside, O-quercetin and rutin can be expected in high levels in CDC Striker seed coats compared to Orb.

5.5 Discussion

The main objective of this study was to investigate the underlying biochemical and genetic control mechanism of green cotyledon bleaching in field pea. CDC Striker (bleaching resistant) and Orb (bleaching susceptible), as well as a RIL population derived from a cross between these two cultivars had been characterized for bleaching resistance under field conditions (Ubayasena et al. 2010). Initial investigations revealed very similar patterns in bleaching resistance under growth chamber conditions as obtained from field grown plants. This indicated that the seeds of growth chamber grown plants could be used to study the underlying biochemical and genetic mechanisms of bleaching resistance.

Photosynthetic pigment (Chl-a, b and total carotenoids) concentration at 35 DAF (physiological maturity) were significantly higher in Orb compared to CDC Striker in both seed coats and cotyledons. McCallum et al. (1997) also reported a higher pigment concentration in a bleaching susceptible cultivar compared to a bleaching resistant cultivar. They left mature seeds on the plants without watering for another 20 days to observe bleaching and reported ‘OSU442-15’ retained greater pigment concentration compared to ‘Promo’ at 55 DAF, similar to our observation with bleaching resistant cultivar CDC Striker. This indicates that pigment accumulation per se was not the basis of bleaching resistance. Relatively low chlorophyll a to b ratio in Orb cotyledons at 35 DAF and 3 DAB compared to CDC Striker indicated that the Chl-a

in CDC Striker was more stable than that of Orb. Declining Chl-a to Chl-b ratio during leaf senescence is a measure of the instability of Chl-a (Gross 1991).

Exposure of photosynthetic plant tissues to high intensity light generated highly reactive intermediates and byproducts which triggered production of toxic oxygen species such as superoxide anion radicals (O_2^-), H_2O_2 and hydroxyl radicals (OH-) (Mehler, 1951; Foyer et al. 1994; Niyogi, 1999). Plants adapt in many ways to protect against photooxidation by dissipation of excess light energy as heat (Demmig-Adams, 1990; Demmig-Adams and Adams, 1992, 1996), alternative electron transport pathways (Mehler, 1951; Artus et al. 1986; Asada, 1999), chloroplast antioxidant systems (Baker and Bowyer, 1994; Foyer et al. 1994; Polle, 1997) and repair of photosystem II (Aro et al. 1993).

Carotenoids play an important role in protecting chlorophyll pigments from bleaching caused by high light intensities (Anderson and Robertson, 1960; Sandmann et al. 1993). However, relatively low concentrations of total carotenoids were observed in the cotyledons and seed coats of CDC Striker compared to Orb during seed developmental stages. In contrast, the concentration of total carotenoids in the cotyledons and seed coats of CDC Striker were significantly higher than Orb after exposure of seeds to accelerated bleaching conditions for 3 days and thereafter. The change of carotenoid composition after exposure to light could be due to the degradation of light sensitive carotenoid derivatives from the total carotenoid pool in the seed tissues (Steiger et al. 1999). The low rate of degradation of total carotenoids observed in both cotyledons and seed coats in CDC Striker compared to Orb during the bleaching periods indicated that the bleaching resistant cultivar may accumulate more light stable carotenoids than the bleaching susceptible cultivar. In addition to quenching excessive light, carotenoids in seeds are important as antioxidants to scavenge free radicals and protect membranes from lipid peroxidation by inhibiting peroxidase activity (Havaux et al. 1991; Havaux, 1998; Pinzino et al. 1999; Calucci et al. 2004). Quantification of carotenoid types may further elucidate the key pigments involved in bleaching resistance.

CDC Striker and bleaching resistant RILs had higher levels of biochemical compounds with UV absorbance maximum at 326 nm compared to Orb and bleaching susceptible RILs. Further characterization of this peak was not conducted, however, the most commonly found natural compounds with UV absorption maximum at wavelengths in this region are flavanones (280 – 290 nm), flavones (304 – 350 nm) and flavonols (352 – 385nm) (Harborne et al. 1975).

These compounds are often found in plant tissues as colorless intermediates of the biosynthesis of phenolic compounds (Ibrahim and Abul-Hajj, 1990; Malikov and Yuldashev, 2002; de Lourdes Mata Bilbao et al. 2007). Often seed coats have high concentrations of phenolic compounds including glycoside forms of flavan-3-ols, flavonols and flavones (Dueñas et al. 2002; Dueñas et al. 2003). The presence of flavonoids in the seed coats of lentils and peas were characterized (Dueñas et al. 2006). High levels of antioxidant activity in pea seeds due to the presence of polyphenolic compounds, mainly hydroxycinnamic compounds, was also reported (Dueñas et al. 2007).

The gene expression experiments conducted in this study showed that the overall gene expression pattern of the seed coats of bleaching resistant and susceptible cultivars are significantly different. Of the differentially expressed transcripts, BURP domain-containing proteins had significantly higher expression in the seed coats of CDC Striker than Orb at all three developmental stages. The BURP domain-containing protein family has diverse functions including early stage zygotic embroyogenesis in field bean (Bassuner et al. 1998; Chesnokov et al. 2002), regulating pectin metabolism by limiting solubilization and depolymerization of pectin in ripening tomato (Zheng et al. 1992; Watson et al. 1994; Zheng et al. 1994) and various roles in seed development in soybean (Batchelor et al. 2002), rice (Wang et al. 2003) and Arabidopsis (Son et al. 2009). Mullin and Xu (2001) showed that water permeability of soybean seed coats decreased with increased amounts of hemicellulose and lower pectin concentration. Compositional differences which result in structural differences of seed coats could reduce the permeability of the CDC Striker seed coats to gas and water and reduce the oxygen dependant chlorophyll degradation in the cotyledons.

The gene expression profile of Orb seed coats indicated high expression of genes responsible for the production of photosynthetic and chlorophyll related metabolic functions, and carbohydrate and amino acid metabolism which coincided with the higher pigment concentration of Orb compared to CDC Striker during seed developmental stages. Furthermore, the up regulated genes involved in membrane integrity, programmed cell death, photoproctection of chlorophyll, stress signaling and cellular redox potential of CDC Striker seed coats coincided with the protection of seed coat membranes and chlorophyll pigments from photo oxidation. These results further suggest that the bleaching resistance trait of CDC Striker may not be due to

the accumulation of more chlorophyll pigments in the cotyledons but effective protection mechanisms of the seed coats.

KEGG pathway analysis also supported the higher accumulation of Chl-a and b in Orb seed coats at 14 and 21 DAF stages, with significant up regulation of genes responsible in porphyrin and chlorophyll metabolism. The transcription study further supported the higher concentration of total carotenoids in the seed coats of Orb compared to CDC Striker by significantly up regulated genes involved in the carotenoid biosynthesis. However, a putative fibrillarin family gene involved in the carotenoid biosynthesis pathway was up regulated at 28 DAF in CDC Striker. This suggests greater accumulation of 3,4 dihydrospheroidene, spheroidene and spifilloxanthin in CDC Striker seed coats compared to Orb. Roszak et al. (2004) suggested that both 3,4 dihydrospheroidene and spheroidene carotenoid derivatives could effectively incorporate into the "gate keeper" protein and then to the reaction centers. Formation of these specific molecular structures is vital for protection of chlorophyll from the reactive singlet state oxygen produced by excessive light.

The KEGG pathway analysis further revealed that the genes responsible for the production of a series of secondary metabolites through flavonoid, flavones and flavonol biosynthesis pathways which are responsible for antioxidant properties in plants tissues such as epiafzelechin, epicatechin, epigallocatechin, kaempferide, kaempferol 3-O-β-D-sophorotrioside, O-quercetin and rutin were significantly up-regulated in seed coats of CDC Striker compared to Orb.

The overall results of biochemical and gene expression studies revealed that the protection of chlorophyll pigments from light mediated bleaching in CDC Striker could be due to the presence of effective carotenoid derivatives and the presence of phenolic antioxidant compounds for effective scavenging of free radical oxygen molecules. Polysaccharides and other polyphenolic compounds which could alter seed coat structures for gas and water permeability may also have a significant effect on the bleaching resistance phenotype. Five main genes, putative fibrillarin family gene (PSOLI04064), BURP domain-containing proteins (PSOLI02815), phosphonopyruvate decarboxylase-like protein (PSOLI03095), hydroxydihydrodaidzein reductase (PSOLI00578) and UDP-glucose glucosyltransferase (PSOLI01493) which could have direct effects on the bleaching resistance trait have been identified. Confirmation of gene expression results using a secondary approach such as

quantitative reverse transcription polymerase chain reaction (qRT-PCR) to validate microarray data is recommended.

CHAPTER 6

6. General discussion, conclusions and future research

Visual quality is one of the major factors that determine the end-use and market value of field pea. Inadequate visual quality may disqualify crops from lucrative markets or result in grading losses. Plant breeding and proper post-harvest storage are important factors in improving visual quality for customers. One of the main constraints faced by pulse breeders in improving the visual quality characteristics of field pea is the lack of knowledge about the genetic control of these traits. These traits are hypothesized to display quantitative inheritance which is strongly influenced by environmental factors. Such quantitative inheritance is often the result of multiple gene segregation. Identification and characterization of the genetic and environmental control of these traits is vital for effective plant breeding. Green cotyledon bleaching resistance in green peas, seed color in yellow peas and seed shape and seed dimpling in both yellow and green types are among the major traits that determine the market quality of field pea.

The objective of the research project was to determine the genetic basis and identify the QTLs for these four visual quality traits affecting field pea market value. This knowledge will assist breeders to improve their strategies for improving visual quality. A further objective of this research was to increase knowledge related to cotyledon bleaching in green pea at the biochemical and gene expression levels. This knowledge will allow for future development of gene based molecular markers to assist in parent and progeny selection for improved bleaching resistant green pea cultivars.

The four visual quality traits evaluated all displayed quantitative inheritance with moderate to high heritability. Their frequency distributions within the investigated RIL populations indicated continuous distribution with transgressive segregation. The occurrence of transgressive segregation in genetically fixed lines (RILs) provided evidence that these traits are under additive genetic control and that the alleles were contributed from both parents or genotype X genotype (G X G) interactions (deVicente and Tanksley 1993; Orf et al. 1999; Timmerman-Vaughan et al. 2002). This study also highlighted the use of appropriate

phenotyping strategies to increase the heritability estimates and thereby make early generation selection more effective (Chapter 3). Screening parents for the presence of favorable alleles and utilizing genetically diverse parents to create segregating populations will result in maximum genetic gain in breeding programs.

Genetic improvement of seed quality attributes in many crop species through recurrent selection has been reported (McPhee 2007). Selecting simultaneously for all these visual quality traits with additive genetic effects with moderate to high heritability could result in rapid genetic progress. Implementation of multiple trait selection such as index selection for seed related quantitative traits is limited due to the complicated genetic nature of the seeds which is often characterized by direct additive effects, cytoplasmic effects and maternal additive effects (Rajcan et al. 2002; van Sanford and Matzinger 1982; Allen 2005). Zhang et al. (2009) proposed two selection indices that could be used in simultaneous selection of seed quality traits in various environments. They proposed a general selection index which could select stable cultivars over multiple environments by utilizing combined information of direct additive, cytoplasmic and maternal additive effects as well as an interaction selection index which combines all three effects, together with the G X E interaction to select special lines in specific environments. In addition, large additive X additive epistatic variance is the genetic basis of most of the economically important traits in self-pollinating crop species (Barker 1984). This kind of epistatic effect could be due to the interaction among different gene products controlling the same trait (Wright 1980). Therefore, further research is needed to fully understand the genetic system of these visual quality traits including any interactions among these additive genetic factors and investigate the genetic control in different genetic backgrounds.

Development of molecular markers utilizing high density molecular maps and QTL mapping is required for marker-assisted selection to improve visual quality for field pea food markets. Development of two genetic linkage maps using AFLP and SSR molecular markers based on two RIL populations derived from green and yellow cotyledon type peas has been described in this study. These maps spanned over 890 cM and 450 cM, respectively. Multiple QTL mapping analysis revealed several QTLs associated with the four visual quality traits flanked with molecular markers. Eight QTLs which were consistent in at least two of the four experimental environments, located on LG II, LG IV, LG V and LG VII, were associated with these four visual quality traits in field pea. In addition to these major QTLs, several location- and

year- specific QTLs were also detected. These could be due to QTL X environment interaction, or lack of detection of QTLs in other environments due to high error variance (Bernardo 2008). The flanking markers to the identified QTLs could be used in marker assisted selection after further validation in a different genetic background.

The QTLs with major effect on visual quality traits could be utilized in field pea breeding by pyramiding into elite cultivars with the help of molecular markers or using phenotypic selection (Bernardo 2008). Complex quantitative traits controlled by many genes with small effects are often challenging to pyramid into one elite cultivar due to the involvement of a large number of QTLs, as well as the inconsistency of estimating the QTL effects in subsequent populations after hybridization and marker assisted selection (Bernardo 2008). Marker assisted recurrent selection (MARS), which involves multiple cycles of marker assisted selection in successive generations, is an approach which could overcome these difficulties (Eathington et al. 2007).

The use of SSR and AFLP markers found associated with QTLs for visual quality traits are limited in marker assisted breeding programs (Jones et al. 1997). This is mainly due to the inherent limitations of these marker systems for automation capabilities and reproducibility. Development of high throughput and reliable markers, such as TaqMan® or KASPar®, based on these detected SSR and AFLP markers may enable pea breeders to use these identified QTLs in developing new cultivars with improved quality.

Despite the comprehensive evaluation of the phenotypes associated with the visual quality traits in field pea, insufficient marker coverage on the linkage maps limited the identification of QTLs on some linkage groups in both populations investigated. In addition, accurate information regarding the genetic distance between the markers and identified QTLs are also needed for those flanking markers to be effectively utilized in marker assisted selection (Van Ooijen 1992; Visscher et al. 1996). The most abundant, reliable and high throughput, multiplexed marker system, i.e., Single Nucleotide Polymorphism (SNP), has become the marker of choice in most large-scale, contemporary mapping and QTL analyses (Gore et al. 2009; Hyten et al. 2008). Therefore it is important to implement the SNP markers on these two populations to develop high density linkage maps and refine the identified QTL regions with much closer, user friendly markers. This will also potentially align the two genetic linkage maps to form a consensus map and to identify more closely linked markers than the ones identified in this study.

This study demonstrated that seed coat characteristics play a significant role in protecting green pea cotyledons from light mediated bleaching. The biochemical study suggested that the accumulation of photosynthetic pigments in the cotyledons up to the seed maturation stage has less influence on bleaching resistance than the rate of degradation when cotyledons are exposed to light. The bleaching resistant cultivar CDC Striker had a slower rate of chlorophyll degradation in cotyledons and a higher carotenoid to chlorophyll ratio in seed coats, than the bleaching susceptible cultivar Orb when seed samples were exposed to high intensity light. However, the carotenoid concentration was greater in the cotyledons of Orb compared to CDC Striker. Carotenoids with specific functional groups play important roles in quenching of excessive light, scavenging free radicals and protecting membranes from lipid peroxidation (Calucci et al. 2004). Therefore, further research to investigate carotenoid profiles with respect to bleaching resistant and susceptible phenotypes and segregating populations would be beneficial to fully understand the role of different carotenoids in controlling this trait. In addition to the carotenoids, involvement of phenolic substances with high level antioxidant activity in pea seed coats were predicted in association with the bleaching resistant phenotype (Chapter 5).

The gene expression profiles of CDC Striker and Orb seed coats at different developmental stages clearly indicated that the expression of genes involved in the production and accumulation of secondary metabolites were significantly different between these cultivars. These secondary metabolites are mainly from the intermediate metabolic products of the flavonoid, flavones and flavonol biosynthesis pathways which have proven antioxidant capabilities and CDC Striker seed coats had greater accumulation owing to the observed high transcriptional activities of the genes involved in these pathways. In addition, the gene expression study also provided validation for the observed differences of the photosynthetic pigment accumulation in the seed coats of Orb and CDC Striker through the transcriptional differences of the porphyrin and chlorophyll pathway and carotenoids biosynthesis pathways (Chapter 5).

As this is the first reported attempt to study the biochemical and genomic regulation of green cotyledon bleaching resistance in field pea, it appeared that the protection mechanism could be due to seed coat structural differences such as permeability to gas and water, as well as chemical differences including the presence of antioxidant phenolic compounds and specific carotenoids. However, this has been validated only by the microarray analysis of

two field pea cultivars. Confirmation of the transcriptional differences of the identified genes with potential impact on bleaching resistance using RT-PCR and further evaluating these gene activities in RILs derived from these two cultivars with known phenotype (Chapter 3) remain to be studied.

The biochemical and gene expression studies reported in this thesis suggested that the protective activity against photooxidative chlorophyll bleaching in cotyledons could be the presence of antioxidant compounds which scavange free radical oxygens produced by triplet chlorophyll formation under high light intensity conditions.

Finally, the concluding remarks on the three hypotheses tested in this thesis are:

1. Accepted the first hypothesis. Cotyledon bleaching resistance in green pea, seed color in yellow pea, seed shape, and seed dimpling in both green and yellow pea types were controlled by multiple genes and their expression is influenced by environmental factors.

2. Accepted the second hypothesis. The field pea RIL populations Orb X CDC Striker and Alfetta X CDC Bronco developed in this research segregated for seed shape and seed dimpling, the Orb X CDC Striker population segregated for cotyledon bleaching resistance, and the Alfetta X CDC Bronco population segregated for seed color, and genetic maps were constructed utilizing molecular marker linkage information. Several regions of the pea genome controlling these visual quality traits were identified using linked molecular markers and phenotypic variability of the RILs.

3. Accepted the third hypothesis. Chemical and physical properties of the seed coat were important factors determining green cotyledon bleaching resistance, these properties were under genetic control, and were characterized by gene expression differences between CDC Striker (bleaching resistant) and Orb (bleaching susceptible).

REFERENCES

Ajmone Marsan, P., Gorni, C., Chittò, A., Redaelli, R., van Vijk, R., Stam, P., and Motto, M. 2001. Identification of QTLs for grain yield and grain-related traits of maize (*Zea mays* L.) using an AFLP map, different testers, and cofactor analysis. Theor. App. Genet. **102**(2): 230-243.

Alba, R., Fei, Z., Payton, P., Liu, Y., Moore, S.L., Debbie, P., Cohn, J., D'Ascenzo, M., Gordon, J.S., Rose, J.K.C., Martin, G., Tanksley, S.D., Bouzayen, M., Jahn, M.M., and Giovannoni, J. 2004. ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. Plant J. **39**(5): 697-714.

Allen, J.O. 2005. Effect of teosinte cytoplasmic genomes on maize phenotype. Genetics **169**: 863-880.

Ameny, M.A., and Wilson, P.W. 1997. Relationship between Hunter color values and beta-carotene contents in white-fleshed African sweet potatoes (*Ipomoea batatas* Lam). J. Sci. Food Agric. **73**(3): 301-306.

Ambrose, M. 2008. Garden Pea. *In* Vegetables II: Fabaceae, Liliaceae, Umbelliferae, and Solanaceae. *Edited by* J. Prohens, and F. Nuez. Springer, New York.. pp. 3-26.

Ambrose, M.J., Wang, T.L., Cook, S.K., and Hedley, C.L. 1987. An analysis of seed development in *Pisum sativum*: IV. Cotyledon cell populations in *vivo* and in *vitro*. J. Exp. Bot. **38**(11): 1909-1920.

Anderson, I.C., and Robertson, D.S. 1960. Role of carotenoids in protecting chlorophyll from photodestruction. Plant Physiol. **35**: 531.

Arens, D., W. Seilmeier, F., Weber, G., Kloos, G., and Grosch, W. 1973. Purification and properties of a carotene co-oxidizing lipoxygenase from peas. Biochem. Biophys. Acta **327**: 295-305.

Arnheim, N., Li, H., and Cui, X. 1991. Genetic mapping by single sperm typing. Animal Genetics, **22**(2): 105-115.

Aro, E.M., Virgin, I., and Andersson, B. 1993. Photoinhibition of photosystem II. Inactivation, protein damage and turnover. Biochim. Biophys. Acta, **1143**: 113.

Artus, N.N., Somerville, S.C., and Somerville, C.R. 1986. The biochemistry and cell biology of photorespiration. CRC Crit. Rev. Plant Sci. **4**: 121.

Asada, K. 1999. The water-water cycle. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50**: 601.

Asokanthan, P. S., Johnson, R. W., Griffith, M., and Krol, M. 1997. The photosynthetic potential of canola embryos. Physiol. Planta. **101**(2): 353-360.

Athwal, G. S., Huber, J. L., and Huber, S.C. 1998. Phosphorylated nitrate reductase and 14-3-3 proteins. Plant Physiol. **118**(3): 1041-1048.

Aubert, G., Morin, J., Jacquin, F., Loridon, K., Quillet, M., Petit, A., Rameau, C., Lejeune-Hénaut, I., Huguet, T., and Burstin, J. 2006. Functional mapping in pea, as an aid to the candidate gene selection and for investigating synteny with the model legume *Medicago truncatula*. Theor. App. Genet. **112**(6): 1024-1041.

Barker, R.J. 1984. Quantitative genetics principles in plant breeding. *In* Gene manipulation in plant improvement. *Edited by* J.P. Gustafson. Plenum Press, New York. pp. 147-176.

Bassam, B.J., Caetano-Anollés, G., and Gresshoff, P.M. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. **196**(1): 80-83.

Bassuner, R., Baumlein, H., Huth, A., Jung, R., Wobus, U., Rapoport, T.A., Saalbach, G., and Muntz, K. 1998. Abundant embryonic mRNA in field bean (*Vicia faba* L.) codes for a new class of seed proteins: cDNA cloning and characterization of the primary translation product. Plant Mol. Biol. **11**: 321 - 334.

Batchelor, A.K., Boutilier, K., Miller, S.S., Hattori, J., Bowman, L.A., Hu, M., Lantin, S., Johnson, D.A., and Miki, B.L. 2002. SCB1, a BURP-domain protein gene, from developing soybean seed coats. Planta, **215**: 523 - 532.

Becker, J.D., Boavida, L.C., Carneiro, J., Haury, M., and Feijo, J.A. 2003. Transcriptional profiling of arabidopsis tissues reveals the unique characteristics of the pollen transcriptome. Plant Physiol. **133**(2): 713-725.

Ben Ze'en, N., and Zohary, D. 1973. Species relationship in the genus *Pisum* L. Isr. J. Botany, **22**: 73-91.

Bennett, M.D., and Leitch, I.J. 2005. Plant DNA C-values Database. (Release 4.0, Oct. 2005) http://www.kew.org/cval/.

Bernardo, R. 2008. Molecular markers and selection for complex traits in plants: Learning from the last 20 Years. Crop Sci. **48**(5): 1649-1664.

Bhattacharyya, M., Martin, C., and Smith, A. 1993. The importance of starch biosynthesis in the wrinkled seed shape character of peas studied by Mendel. Plant Mol. Biol. **22**(3): 525-531.

Blixt, S. 1958. Cytology of Pisum II. The normal karyotpe. Agric. Hort. Genet. **16**: 221-237.

Blixt, S. 1959. Cytology of Pisum III. Investigation of five interchange lines and coordination of linkage groups with chromosomes. Agric. Hort. Genet. 17: **47**-75.

Blixt, S. 1962. Studies in induced mutations. VI. Mutations in seed color, flower color, maculum color, pod color and grey spotting of leaves. Acta Hort. Genet. **20**: 95-100.

Blixt, S. 1972. Mutation genetics of Pisum. Agric. Hort. Genet. **30**: 1-293.

Boeswinkel, F.D., and Bouman, F. 1995. The seed: Structure and function. *In* Seed development and germination. *Edited by* J. Kigel, and G. G. Marcel Dekker, New York. pp. 1-24.

Borisjuk, L., Nguyen, T.H., Neuberger, T., Rutten, T., Tschiersch, H., Claus, B., Feussner, I., Webb, A.G., Jakob, P., Weber, H., Wobus, U., and Rolletschek, H. 2005. Gradients of lipid storage, photosynthesis and plastid differentiation in developing soybean seeds. New Phytol. **167**(3): 761-776.

Borisjuk, L., Rolletschek, H., Radchuk, R., Weschke, W., Wobus, U., and Weber, H. 2004. Seed development and differentiation: A role for metabolic regulation. Plant Biol. **6**(4): 375-386.

Borisjuk, L., Walenta, S., Rolletschek, H., Mueller-Klieser, W., Wobus, U., and Weber, H. 2002. Spatial analysis of plant metabolism: Sucrose imaging within *Vicia faba* cotyledons reveals specific developmental patterns. Plant J. **29**(4): 521-530.

Bowren, K.E., and Cooke, D.A. 1975. Effects of legumes in cropping systems in northeastern Saskatchewan. Can. J. Plant Sci. **55**:351.

Brazma, A., Hingamp, P., Quackenbush, J., Sherlock, G., Spellman, P., Stoecket, C., Aach, J., Ansorge, W., Ball, C.A., Causton, H.C., Gaasterland, T., Glenisson, P., Holstege, F.C.P., Kim, I.F., Markowitz, V., Matese, J.C., Parkinson, H., Robinson, A., Sarkans, U., Schulez-Kremer, S., Stewart, J., Taylor, R., Vilo, J., and Vingron, M. 2001. Minimum information about a micro-array experiment (MIAME) - toward standards for microarray data. Nat. Genet. **29**: 365-371.

Brenner, M.L., and Cheikh, N. 1995. The role of hormones in photosynthate partitioning and seed filling. *In* Plant Hormones, physiology, biochemistry and molecular biology. *Edited by* P.J. Davis. Kluwer Acamemic Publishers, Dordrecht, Netherlands. pp. 649-670.

Buitink, J., Leger, J.J., Guisle, I., Vu, B.L., Wuillème, S., Lamirault, G., Bars, A.L., Le Meur, N., Becker, A., Küster, H., and Leprince, O. 2006. Transcriptome profiling uncovers metabolic and regulatory processes occurring during the transition from desiccation-sensitive to desiccation-tolerant stages in *Medicago truncatula* seeds. Plant J. **47**(5): 735-750.

Calucci, L., Capocchi, A., Galleschi, L., Ghiringhelli, S., Pinzino, C., Saviozzi, F., and Zandomeneghi, M. 2004. Antioxidants, free radicals, storage proteins, puroindolines, and proteolytic activities in bread wheat (*Triticum aestivum*) seeds during accelerated aging. J. Agric. Food Chem. **52**(13): 4274-4281.

Canadian Grain Commission, 2008. Official Grain Grading Guide. 1 August 2008. Canadian Grain Commission, Winnipeg, Man. pp. 343-360.

Carlson, J.B. 1973. Morphology. *In* Soybeans: Improvement, production and uses. *Edited by* J.R. Wilcox. The American Society of Agronomy, Inc. pp. 17-95.

Cernac, A., and Benning, C. 2004. *WRINKLED1* encodes an AP2/EREB domain protein involved in the control of storage compound biosynthesis in Arabidopsis. Plant J. **40**(4): 575-585.

Cheng, M., McPhee, K.E., and Baik, B.-K. 2004. Bleaching of green peas and changes in enzyme activities of seeds under simulated climatic conditions. J. Food Sci. **69**(7): 511-518.

Chesnokov, Y., Meister, A., and Manteuffel, R. 2002. A chimeric green fluorescent protein gene as an embryonic marker in transgenic cell culture of **Nicotiana plumbaginifolia** V iv. Plant Sci. **162**: 59 - 77.

Cober, E.R., Voldeng, H.D., and Fregeau-Reid, J.A. 1997. Heritability of seed shape and seed size in soybean. Crop Sci. **37**(6): 1767-1769.

Cogan, N.O.I., Smith, K.F., Yamada, T., Francki, M.G., Vecchies, A.C., Jones, E.S., Spangenberg, G.C., and Forster, J.W. 2005. QTL analysis and comparative genomics of herbage quality traits in perennial ryegrass (*Lolium perenne* L.). Theor. App. Genet. **110**(2): 364-380.

Craig, S., Goodchild, D.L., and Hardham, A.R. 1979. Structural aspects of protein accumulation in developing pea cotyledons. I. Qualitative and quantitative changes in parenchyma cells vacuoles. Aust. J. Plant Physiol. **6**: 81-98.

De Bodt, S., Raes, J., Van de Peer, Y., and Theissen, G. 2003. And then there were many: MADS goes genomic. Trends Plant Sci. **8**(10): 475-483.

Decker, E. A. 1997. Phenolics: prooxidents or antioxidents ? Nutr. Rev. **55**: 396-407.

de Lourdes Mata Bilbao, M., Andrés-Lacueva, C., Jáuregui, O., and Lamuela-Raventós, R.M. 2007. Determination of flavonoids in a Citrus fruit extract by LC-DAD and LC-MS. Food Chem. **101**(4): 1742-1747.

Del, B., Frohberg, Stack, Berzonsky, and Kianian. 2003. Detection of QTL linked to *Fusarium* head blight resistance in *Sumai 3*-derived North Dakota bread wheat lines. Theor. App. Genet. **106**(6): 1027-1031.

Demmig-Adams, B. 1990. Carotenoids and photoprotection in plants: a role for the xanthophyll zeaxanthin. Biochim. Biophys. Acta, **1020**: 1-24.

Demmig-Adams, B., Adams, W.W., Barker, D.H., Logan, B.A., Bowling, D.R., and Verhoeven, A.S. 1996. Using chlorophyll fluorescence to assess the fraction of absorbed light allocated to thermal dissipation of excess excitation. Physiol. Plant. **98**: 253.

Demmig-Adams, B., and Adams, W.W. 1992. Photoprotection and other responses of plants to high light stress. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43**: 599.

Demmig-Adams, B., and Adams, W.W. 1996. The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci. **1**: 21.

Desprez, T., Amselem, J., Caboche, M., and Höfte, H. 1998. Differential gene expression in Arabidopsis monitored using cDNA arrays. Plant J. **14**(5): 643-652.

deVicente, M.C., and Tanksley, S.D. 1993. QTL analysis of transgressive segregation in an interspecific tomato cross. Genetics, **134**(2): 585-596.

Dirlewanger, E., Isaac, P.G., Ranade, S., Belajouza, M., Cousin, R., and Vienne, D. 1994. Restriction fragment length polymorphism analysis of loci associated with disease resistance genes and developmental traits in *Pisum sativum* L. Theor. App. Genet. **88**(1): 17-27.

Domoney, C., Duc, G., Ellis, T.H.N., Ferrándiz, C., Firnhaber, C., Gallardo, K., Hofer, J., Kopka, J., Küster, H., Madueño, F., Munier-Jolain, N.G., Mayer, K., Thompson, R., Udvardi, M., and Salon, C. 2006. Genetic and genomic analysis of legume flowers and seeds. Curr. Opin. Plant Biol. **9**(2): 133-141.

Dondrup, M., Goesmann, A., Bartels, D., Kalinowski, J., Krause, L., Linke, B., Rupp, O., Sczyrba, A., Pühler, A., and Meyer, F. 2003. EMMA: a platform for consistent storage and efficient analysis of microarray data. J. Biotech. **106**(2-3): 135-146.

Donson, J., Fang, Y., Espiritu-Santo, G., Xing, W., Salazar, A., Miyamoto, S., Armendarez, V., and Volkmuth, W. 2002. Comprehensive gene expression analysis by transcript profiling. Plant Mol. Biol. **48**(1): 75-97.

Dribnenki, P.C. 1979. A study of bleaching resistance in green cotyledon, dry edible peas., M. Sc. Thesis, Department of Crop Science, University of Saskatchewan, Sask.

Dueñas, M., Hernández, T., and Estrella, I. 2002. Phenolic composition of the cotyledon and the seed coat of lentils (*Lens culinaris* L.). Eur. Food Res. Technol. **215**(6): 478-483.

Dueñas, M., Hernández, T., and Estrella, I. 2006. Assessment of in vitro antioxidant capacity of the seed coat and the cotyledon of legumes in relation to their phenolic contents. Food Chem. **98**(1): 95-103.

Dueñas, M., Hernández, T., and Estrella, I. 2007. Influence of the action of exogenous enzymes on the polyphenolic composition of pea: Effect on the antioxidant activity. Eur. Food Res. Technol. **225**(3): 493-500.

Dueñas, M., Sun, B., Hernández, T., Estrella, I., and Isabel, S.M. 2003. Proanthocyanidin Composition in the Seed Coat of Lentils (*Lens culinaris* L.). J Agric. Food Chem. **51**(27): 7999-8004.

Dziedzic, S. Z., and Hudson, B. J. F. 1983. Polyhydroxy chalcones and flavanones as antioxidants for edible oils. Food Chem. **12**(3): 205-212.

Eathington, S.R., Crosbie, T.M., Edwards, M.D., Reiter, R.S., and Bull, J.K. 2007. Molecular markers in a commercial breeding program. Crop Sci. **47**(S3):S154-S165.

Eckhardt, U., Grimm, B., and Hörtensteiner, S. 2004. Recent advances in chlorophyll biosynthesis and breakdown in higher plants. Plant Mol. Biol. **56**(1): 1-14.

Edelenbos, M., Christensen, L.P., and Grevsen, K. 2001. HPLC determination of chlorophyll and carotenoid pigments in processed green pea cultivars (*Pisum sativum* L.). J. Agric. Food Chem. **49**(10): 4768-4774.

Edge, R. and Truscott, T. G. 1999. Carotenoid radicals and the interaction of carotenoids with active oxygen species. *In*. Advances in photosynthesis: the photochemistry of carotenoids. *Edited by* H. A. Frank, A. J. Young, G. Britton, and R. J. Cogdell, Vol 8. Kluwer, Dordrecht. pp. 223-234

El Yahyaoui, F., Kuster, H., Ben Amor, B., Hohnjec, N., Puhler, A., Becker, A., Gouzy, J., Vernie, T., Gough, C., Niebel, A., Godiard, L., and Gamas, P. 2004. Expression profiling in *Medicago truncatula* identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. Plant Physiol. **136**(2): 3159-3176.

Ellis, T.H.N., Hellens, R.P., Turner, L., Lee, C., Domoney, C., and Welham, T. 1993. On the pea linkage map. Pisum Genet. **25**: 5-12.

Ellis, T.H.N., Turner, L., Hellens, R.P., Lee, D., Harker, C.L., Enard, C., Domoney, C., and Davies, D.R. 1992. Linkage maps in pea. Genetics, **130**(3): 649-663.

Emery, R.J.N., and Atkins, C.A. 2006. Cytokinins and seed development. *In* Seed science and technology: Trends and advances. *Edited by* A.S. Basra. Haworth Press Inc., Binghamton, New York. pp. 63-93.

Erlich, H.A., Gelfand, D., and Sninsky, J.J. 1991. Recent advances in the polymerase chain reaction. Science, **252**(5013): 1643-1651.

Eujayl, I., Baum, M., Powell, W., Erskine, W., and Pehu, E. 1998. A genetic linkage map of lentil (*Lens* sp.) based on RAPD and AFLP markers using recombinant inbred lines. Theor. App. Genet. **97**(1): 83-89.

FAO. 2008. FAOSTAT. ProdSTAT.Crops [online]. Available from http://faostat.fao.org/site/567/Desktop.Default.aspx?PageID=567 [accessed 1 August 2009].

Fatokun, C.A., Menancio-Hautea, D.I., Danesh, D., and Young, N.D. 1992. Evidence for orthologous seed weight genes in cowpea and mung bean based on RFLP mapping. Genetics, **132**(3): 841-846.

Feierabend, J., and Schubert, B. 1978. Comparative investigation of the action of several chlorosis-inducing herbicides on the biogenesis of chloroplasts and leaf microbodies. Plant Physiol. **61**(6): 1017-1022.

Firnhaber, C., Pühler, A., and Küster, H. 2005. EST sequencing and time course microarray hybridizations identify more than 700 *Medicago truncatula*; genes with developmental expression regulation in flowers and pods. Planta, **222**(2): 269-283.

Flavell, R. 2010. From genomics to crop breeding. Nat. Biotech. **28**(2): 144-145.

Foyer, C.H., Descourvires, P., and Kunert, K.J. 1994a. Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. Plant Cell Environ. **17**: 507.

Foyer, C.H., Lelandais, M., and Kunert, K.J. 1994b. Photooxidative stress in plants. Physiol. Plant. **92**: 696.

Gallardo, K., Firnhaber, C., Zuber, H.l.n., Hélène, D., Belghazi, M., Henry, C.l., Küster, H., and Thompson, R. 2007. A combined proteome and transcriptome analysis of developing *Medicago truncatula* seeds. Mol. Cell. Proteomics, **6**(12): 2165-2179.

Ganeshan, S., Drinkwater, J.M., Repellin, A., and Chibbar, R.N. 2010. Selected carbohydrate metabolism genes show coincident expression peaks in grains of in vitro-cultured immature spikes of wheat (*Triticum aestivum* L.). J. Agric. Food Chem. **58**(7): 4193-4201.

Gawlowska, M., Swiecicki, W., and Wolko, B. 2005. Comparison of genetic maps for two related pea populations (*Pisum sativum* L.). Pisum Genet. **37**: 19-23.

Girke, T., Todd, J., Ruuska, S., White, J., Benning, C., and Ohlrogge, J. 2000. Microarray analysis of developing arabidopsis seeds. Plant Physiol. **124**(4): 1570-1581.

Gore, M.A., Wright, M.H., Ersoz, E.S., Bouffard, P., Szekeres, E.S., Jarvie, T.P., Hurwitz, B.L., Narechania, A., Harkins, T.T., Grills, G.S., Ware, D.H., and Buckler, E.S. 2009. Large-scale discovery of gene-enriched SNPs. Plant Gen. **2**(2): 121-133.

Griffiths, M., Sistrom, W.R., Cohen-Bazire, G., and Stanier, R.Y. 1955. Function of carotenoids in photosynthesis. Nature. 176: 1211-1214.

Gross, J. 1991. Pigments in vegetables: Chlorophylls and carotenoids. AVI Books, New York.

Gubbels, G.H., and Ali-Khan, S.T. 1990. Screening green field pea genotypes for resistance to color loss. Can. J. Plant Sci. **70**: 45-50.

Gurevitch, J., and Chester, J.S.T. 1986. Analysis of repeated measures experiments. Ecology, **67**(1): 251-255.

Gutierrez, L., Conejero, G., Castelain, M., Guenin, S., Verdeil, J.-L., Thomasset, B., and Van Wuytswinkel, O. 2006. Identification of new gene expression regulators specifically expressed during plant seed maturation. J. Exp. Bot. **57**(9): 1919-1932.

Hall, K.J., Parker, J.S., and Ellis, T.H.N. 1997a. The relationship between genetic and cytogenetic maps of pea. I. Standard and translocation karyotypes. Genome, **40**: 744-754.

Hall, K.J., Parker, J.S., Ellis, T.H.N., Turner, L., Knox, M.R., Hofer, J.M.I., Lu, J., Ferrandiz, C., Hunter, P.J., Taylor, J.D., and Baird, K. 1997b. The relationship between genetic and cytogenetic maps of pea. II. Physical maps of linkage mapping populations. Genome, **40**: 755-769.

Harborne, J.B., Marby, T.J., and Mabry, H. 1975. The flavonoids. Chapman and Hall, London.

Hatano, T., Miyatake, H., Natsume, M., Osakabe, N., Takizawa, T., Ito, H., and Yoshida, T. 2002. Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects. Phytochem. **59**(7): 749-758.

Havaux, M. 1998. Carotenoids as membrane stabilizers in chloroplasts. Trends Plant Sci. **3**: 147.

Havaux, M., Gruszecki, W.I., Dupont, I., and Leblanc, R.M. 1991. Increased heat emission and its relationship to the xanthophyll cycle in pea leaves exposed to strong light stress. J. Photoch. Photobio. B. **8**(4): 361-370.

Hedley, C.L., Smith, C.M., Ambrose, M.J., Cook, S., and Wang, T.L. 1986. An analysis of seed development in *Pisum sativum* II. The effect of the r-locus on the growth and development of the seed. Ann. Bot. (Lond.), **58**(3): 371-379.

Hegde, P., Qi, R., Abernathy, K., Gay, C., Dharap, S., Gaspard, R., Hughes, J.E., Snesrud, E., Lee, N., and Quackenbush, J. 2000. A concise guide to cDNA microarray analysis. Biotechniques, **2000**(29): 548-556.

Hennig, L., Gruissem, W., Grossniklaus, U., and Kohler, C. 2004. Transcriptional programs of early reproductive stages in Arabidopsis. Plant Physiol. **135**(3): 1765-1775.

Hoeck, J.A., Fehr, W.R., Shoemaker, R.C., Welke, G.A., Johnson, S.L., and Cianzio, S.R. 2003. Molecular marker analysis of seed size in soybean. Crop Sci. **43**(1): 68-74.

Hoff, A. J. 1979. Applications of ESR in photosynthesis. Phys. Rep. **54**(2): 75-200.

Holden, M. 1965. Chlorophyll bleaching by legume seeds. J. Sci. Food Agric. **16**(6): 312-325.

Honys, D., and Twell, D. 2003. Comparative analysis of the Arabidopsis pollen transcriptome. Plant Physiol. **132**(2): 640-652.

Hosfield, G.L. 1991. Genetic control of production and food quality factors in dry bean. Food Technol. **45**(98): 100-103.

Hughes, T.R., Mao, M., Jones, A.R., Burchard, J., Marton, M.J., Shannon, K.W., Lefkowitz, S.M., Ziman, M., Schelter, J.M., Meyer, M.R., Kobayashi, S., Davis, C., Dai, H., He, Y.D., Stephaniants, S.B., Cavet, G., Walker, W.L., West, A., Coffey, E., Shoemaker, D.D., Stoughton, R., Blanchard, A.P., Friend, S.H., and Linsley, P.S. 2001. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. Nat. Biotech. **19**(4): 342-347.

Hyten, D., Song, Q., Choi, I.-Y., Yoon, M.-S., Specht, J., Matukumalli, L., Nelson, R., Shoemaker, R., Young, N., and Cregan, P. 2008. High-throughput genotyping with the Golden Gate assay in the complex genome of soybean. Theor. App. Genet. **116**(7): 945-952.

Hyten, D.L., Pantalone, V.R., Sams, C.E., Saxton, A.M., Landau-Ellis, D., Stefaniak, T.R., and Schmidt, M.E. 2004. Seed quality QTL in a prominent soybean population. Theor. App. Genet. **109**(3): 552-561.

Ibrahim, A.-R., and Abul-Hajj, Y.J. 1990. Microbiological Transformation of (1) Flavanone and (±) Isoflavanone. J. Nat. Prod. **53**(3): 644-656.

Irzykowska, L., Wolko, B., and Swiecicki, W.K. 2001. The genetic linkage map of pea (*Pisum sativum* L.) based on molecular, biochemical and morphological markers. Pisum Genet. **33**: 13-18.

Jansen, R.C. 1992. A general mixture model for mapping quantitative trait loci by using molecular markers. Theor. App. Genet. **85**(2): 252-260.

Jansen, R. C. 1993. Interval mapping of multiple quantitative trait loci. Genetics **135**(1): 205-211.

Jansen, R. C. 1994. Controlling the Type I and Type II errors in mapping quantitative trait loci. Genetics **138**(3): 871-881.

Jansen, R. C., and Stam, P. 1994. High resolution of quantitative traits into multiple loci via interval mapping. Genetics **136**(4): 1447-1455.

Jansen, R. C., Ooijen, J.W., Stam, P., Lister, C., and Dean, C. 1995. Genotype-by-environment interaction in genetic mapping of multiple quantitative trait loci. Theor. App. Genet. **91**(1): 33-37.

Jegadeesan, S., Yu, K., Poysa, V., Gawalko, E., Morrison, M., Shi, C., and Cober, E. 2010. Mapping and validation of simple sequence repeat markers linked to a major gene controlling seed cadmium accumulation in soybean (*Glycine max* L.). Theor. App. Genet.**121**(2): 283-294.

Jensen, J. 1989. Estimation of recombination parameters between a quantitative trait locus (QTL) and two marker gene loci. Theor. App. Genet. **78**(5): 613-618.

Johnson, S., Liu, C.M., Hedley, C.L., and Wang, T.L. 1994. An analysis of seed development in *Pisum sativum* XVIII. The isolation of mutants defective in embryo development. J. Exp. Bot. **45**(11): 1503-1511.

Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, F., van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., Linacero, R., Vazquez, A., and Karp, A. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol. Breeding, **3**(5): 381-390.

Kathleen, W., Helge, K., Ruslana, R., Martin, M., Heiko, W., Aaron, F., Alisdair, R.F., Isolde, S., and Hans, W. 2008. Increasing amino acid supply in pea embryos reveals specific interactions of N and C metabolism, and highlights the importance of mitochondrial metabolism. Plant J. **55**(6): 909-926.

Kirmaier, C., and Holten, D. 1987. Primary photochemistry of reaction centers from the photosynthetic purple bacteria. Photosynth. Res. **13**(3): 225-260.

Knapp, S. J., Bridges, W. C., and Birkes, D. 1990. Mapping quantitative trait loci using molecular marker linkage maps. Theor. App. Genet. **79**(5): 583-592.

Kosterin, O.E. 1993. Genes a and d may not be in the same linkage group. Pisum Genet. **25**: 23-26.

Kosterin, O.E., and Rozov, S.M. 1993. Mapping of the new mutation *blb* and the problem of integrity of linkage. Pisum Genet. **25**: 27-31.

Krieger-Liszkay, A. 2005. Singlet oxygen production in photosynthesis. J. Exp. Bot. **56**(411): 337-346.

Krinsky, N. I. 1971. Function *In* Carotenoids. *Edited by* O. Isler, G. Guttman, and U Solms. Birkhauser Verlag, Basel, pp: 161-716

Kuhn, E. 2001. From library screening to microarray technology: Strategies to determine gene expression profiles and to identify differentially regulated genes in plants. Ann. Bot. **87**(2): 139-155.

Küster, H., and Dondrupup, M. 2006. Integration of gene expression profiling in European projects on legumes. Grain Legumes, **46**: 14-15.

Küster, H., Hohnjec, N., Krajinski, F., El Yahyaoui, F., Manthey, K., Gouzy, J., Dondrup, M., Meyer, F., Kalinowski, J., Brechenmacher, L., van Tuinen, D., Gianinazzi-Pearson, V., Pühler, A., Gamas, P., and Becker, A. 2004. Construction and validation of cDNA-based Mt6k-RIT macro- and microarrays to explore root endosymbioses in the model legume Medicago truncatula. J. Biotechnol. **108**(2): 95-113.

Küster, H., Vieweg, M.F., Manthey, K., Baier, M.C., Hohnjec, N., and Perlick, A.M. 2007. Identification and expression regulation of symbiotically activated legume genes. Phytochemistry, **68**(1): 8-18.

Lamprecht, H. (ed.) 1958. Genetics of *Pisum* [Translated by Amstutz E.]. Califonia Packing, San Leandro, CA.

Lamprecht, H. 1948. The variation of linkage and the course of crossing over. Agric. Hort. Genet. Landskrona, 6: 10-49.

Lamprecht, H. 1957. Die lage der gene Ve and Vim in den chromosomen II Bzw. IV von *Pisum*. Agri. Hort. Genet. Landskrona, **15**: 1-11.

Lamprecht, H. 1959. The inheritance of colors of a seeds of *Pisum*. Agri. Hort. Genet. Landskrona, **17**: 1-18.

Lamprecht, H. 1962. Über ein neues, die Form der Pisum-Samen beeinflussendes Gen sowie ein neues Gen für Teilfarbigkeit. Agr.Hort.Gen. Landskrona, **20**: 137-155.

Lander, E. S., and Botstein, D. 1989. Mapping mendelian factors underlying quantitative traits Using RFLP linkage maps. Genetics **121**(1): 185-199.

Landry, B.S., Hubert, N., Crete, R., Chang, M.S., Lincoln, S.E., and Etoh, T. 1992. A genetic map for *Brassica oleracea* based on RFLP markers detected with expressed DNA sequences and mapping of resistance genes to race 2 of *Plasmodiophora brassicae* (Woronin). Genome, **35**: 409-420.

Landry, B.S., Hubert, N., Etoh, T., Harada, J.J., and Lincoln, S.E. 1991. A genetic map for *Brassica napus* based on restriction fragment length polymorphisms detected with expressed DNA sequences. Genome, **34**: 543-552.

Laucou, V., Haurogné, K., Ellis, T.H.N., and Rameau, C. 1998. Genetic mapping in pea. 1. RAPD-based genetic linkage map of *Pisum sativum*. Theor. App. Genet. **97**(5): 905-915.

Laudencia-Chingcuanco, D., Stamova, B., You, F., Lazo, G., Beckles, D., and Anderson, O. 2007. Transcriptional profiling of wheat caryopsis development using cDNA microarrays. Plant Mol. Biol. **63**(5): 651-668.

Lei, S., Yao, X., Yi, B., Chen, W., Ma, C., Tu, J., and Fu, T. 2007. Towards map-based cloning: fine mapping of a recessive genic male-sterile gene (BnMs2) in *Brassica napus* L. and syntenic region identification based on the *Arabidopsis thaliana*; genome sequences. Theor. App. Genet. **115**(5): 643-651.

Li, J., Båga, M., Rossnagel, B.G., Legge, W.G., and Chibbar, R.N. 2008. Identification of quantitative trait loci for β-glucan concentration in barley grain. J. Cereal Sci. **48**(3): 647-655.

Liang, P., and Pardee, A.B. 1992. Differential display of eukaryotic messenger RNA by means of the Polymerase Chain Reaction. Science, **257**(5072): 967-971.

Lichtenthaler, H.K. (ed.) 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Academic Press, San Diego, CA.

Loria. 1979. Mechanism of increased susceptibility of bleached pea seeds to seed and seedling rot. Phytopathology, **69**(6): 573-575.

Loridon, K., McPhee, K., Morin, J., Dubreuil, P., Pilet-Nayel, M., Aubert, G., Rameau, C., Baranger, A., Coyne, C., Lejeune-Hènaut, I., and Burstin, J. 2005. Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). Theor. App. Genet. **111**(6): 1022-1031.

Luo, Z. W., and Kearsey, M. J. 1989. Maximum likelihood estimation of linkage between a marker gene and a quantitative locus. Heredity, **63**(3): 401-408.

Maguire, J.D., Kropf, J.P., and Steen, K.M. 1973. Pea seed viability in relation to bleaching. Proc. Assoc. Off. Seed Anal. **63**: 51-58.

Mahmoud, S.H., Gatehouse, J.A., and Boulter, D. 1984. Inheritance and mapping of isoenzymes in pea (*Pisum sativum* L.). Theor. App. Genet. **68**(6): 559-566.

Malikov, V.M., and Yuldashev, M.P. 2002. Phenolic Compounds of plants of the Scutellaria genus. Distribution, structure, and properties. Chem. Nat. Compd. **38**(5): 473-519.

Mansur, L.M., Orf, J.H., Chase, K., Jarvik, T., Cregan, P.B., and Lark, K.G. 1996. Genetic mapping of agronomic traits using recombinant inbred lines of soybean. Crop Sci. **36**(5): 1327-1336.

Manthey, K., Krajinski, F., Hohnjec, N., Firnhaber, C., Pühler, A., Perlick, A.M., and Küster, H. 2004. Transcriptome profiling in root nodules and arbuscular mycorrhiza identifies a collection of novel genes induced during *Medicago truncatula* root endosymbioses. Mol. Plant Microbe In. **17**(10): 1063-1077.

Marbach, I., and Mayer, A.M. 1974. Permeability of seed coats to water as related to drying conditions and metabolism of phenolics. Plant Physiol. **54**(6): 817-820.

Marcus, R.T. 1998. The measurement of color. *In* Color for science, art and technology. *Edited by* K. Nassau. Elsevier Science, Armsterdam. pp. 31–96.

Marshall, G.A., Moody, M.W., and Hackney, C.R. 1988. Differences in color, texture, and flavor of processed meat from red swamp crawfish (*Procambarus clarkii*) and white river crawfish (*P. acutus acutus*). J.Food Sci. **53**(1): 280-281.

Matile, P., Hortensteiner, S., and Thomas, H. 1999. Chlorophyll degradation. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50**(1): 67.

Maughan, P.J., Maroof, M.A.S., and Buss, G.R. 1996. Molecular-marker analysis of seed-weight: genomic locations, gene action, and evidence for orthologous evolution among three legume species. Theor. App. Genet. **93**(4): 574-579.

McCallum, J., Timmerman-Vaughan, G., Frew, T., and Russell, A. 1997. Biochemical and genetic linkage analysis of green seed color in field pea. J. Amer. Soc. Hortic. Sci. **122**(2): 218-225.

McCartney, L., and Knox, J.P. 2002. Regulation of growth, development and whole organism physiology. regulation of pectic polysaccharide domains in relation to cell development and cell properties in the pea testa. J. Exp. Bot. **53**: 707-713.

McPhee, K. 2007. Pea. *In* Genome mapping and molecular breeding in plants. Vol. 3. *Edited by* C. Kole. Springer-Verlag, Berlin. pp. 33-47.

Mehler, A.H. 1951. Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. Arch. Biochem. Biophys. **33**: 65.

Mepsted, R., Paul, N.D., Stephen, J., Corlett, J.E., Nogues, S., Baker, N.R., G. Jones, G., and Ayres, P. G. (1996). Effects of enhanced UV-B radiation on pea (*Pisum sativum* L.) grown under field conditions in the UK. Glob. Change. Biol. **2**(4): 325-334.

Meyers, B.C., Galbraith, D.W., Nelson, T., and Agrawal, V. 2004. Methods for transcriptional profiling in plants. Be fruitful and replicate. Plant Physiol. **135**(2): 637-652.

Miyamoto, N., Goto, Y., Matsui, M., Ukai, Y., Morita, M., and Nemoto, K. 2004. Quantitative trait loci for phyllochron and tillering in rice. Theor. App. Genet. **109**(4): 700-706.

Muehlbauer, F.J. 1992. Use of inroduced germplasm in cool season food legume cultivar development, Part 2.. *In* Use of plant introductions in cultivar development. *Edited by* H.L. Shands, and L.E. Weisner. Crop Science Society of America, Madison, WI. pp. 51-76.

Mullin, W.J., and Xu, W. 2001. Study of soybean seed coat components and their relationship to water absorption. J. Agric. Food Chem. **49**(11): 5331-5335.

Munier-Jolain, N., and Ney, B. 1998a. Seed growth rate in grain legumes II. Seed growth rate depends on cotyledon cell number. J. Exp. Bot. **49**(329): 1971-1976.

Munier-Jolain, N., and Ney, B. 1998b. Seed growth rate in grain legumes II. Seed growth rate depends on cotyledon cell number. J. Exp. Bot. **49**: 1971-1976.

Munier-Jolain, N., and Salon, C. 2003. Can sucrose content in the phloem sap reaching field pea seeds (*Pisum sativum* L.) be an accurate indicator of seed growth potential? J. Exp. Bot. **54**(392): 2457-2465.

Munier-Jolain, N., Roche, R., Ney, B., and Duthion, C. 1998. Seed growth rate in grain legumes I. Effect of photoassimilate availability on seed growth rate. J. Exp. Bot. **49**(329): 1963-1969.

Müntz, K., Rudolph, A., Schlesier, G., and Silhengst, P. 1978. The function of the pericarp in fruits of crop legumes. Genet. Resour. Crop Ev. **26**(1): 37-67.

Murfet, I.C., and Reid, J.B. 1993. Peas: genetics, molecular biology and biotechnology. *In* Developmental mutants. *Edited by* R. Casey, and D.R. Davis. CAB International, Wallingford. pp. 165-216.

Natella, F., Nardini, M., Di Felice, M., and Scaccini, C. 1999. Benzoic and cinnamic acid derivatives as antioxidants: Structure-activity relation. J. Agric. Food Chem. **47**(4): 1453-1459.

Ney, B., and Turc, O. 1993. Heat-unit-based description of the reproductive development of Pea. Crop Sci. **33**(3): 510-514.

Ney, B., Duthion, C., and Fontaine, E. 1993. Timing of reproductive abortions in relation to cell division, water content, and growth of pea seeds. Crop Sci. **33**(2): 267-270.

Niyogi, K.K. 1999. Photoprotection revisited: Genetic and molecular approaches. Annu. Rev. Plant Phys. **50**(1): 333-359.

Nleya, T., Vadenberg, A., Araganosa, G., Warkentin, T., Musehlbauer, F.J., and Slinkard, A.E. 2000. Produce quality of food legumes: Genotype (G), environment (E) and (GxE) considerations. *In* Linking Research and Marketing Opportunities for Pulses in the 21st Century. *Edited by* R. Knight. Kluwer Academic Publishers, Netherlands. pp. 173-182.

Olson, M., Hood, L., Cantor, C., and Botstein, D. 1989. A common language for physical mapping of the human genome. Science, **245**(4925): 1434-1435.

Orf, J.H., Chase, K., Jarvik, T., Mansur, L.M., Cregan, P.B., Adler, F.R., and Lark, K.G. 1999. Genetics of soybean agronomic traits: I. Comparison of three related recombinant inbred populations. Crop Sci. **39**(6): 1642-1651.

Ozga, J.A., van Huizen, R., and Reinecke, D.M. 2002. Hormone and seed-specific regulation of pea fruit growth. Plant Physiol. **128**(4): 1379-1389.

Paterson, A.H., Damon, S., Hewitt, J.D., Zamir, D., Rabinowitch, H.D., Lincoln, S.E., Lander, E.S., and Tanksley, S.D. 1991. Mendelian factors underlying quantitative traits in Tomato: Comparison across species, generations, and environments. Genetics, **127**(1): 181-197.

Paterson, A.H., DeVerna, J.W., Lanini, B., and Tanksley, S.D. 1990. Fine mapping of quantitative trait loci using selected overlapping recombinant chromosomes, in an interspecies cross of tomato. Genetics, **124**(3): 735-742.

Paterson, A.H., Lander, E.S., Hewitt, J.D., Peterson, S., Lincoln, S.E., and Tanksley, S.D. 1988. Resolution of quantitative traits into Mendelian factors by using a complete linkage map of restriction fragment length polymorphisms. Nature, **335**(6192): 721-726.

Pfeiffer, T.W., and Egli, D.B. 1988. Heritability of seed-filling period estimates in soybean. Crop Sci. **28**(6): 921-925.

Phillips, R.L., and Vasil, I.K. (eds). 1994. DNA based markers in plants. Kluwer, Dordrecht, The Netherland.

Pilet-Nayel, M.L., Muehlbauer, F.J., McGee, R.J., Kraft, J.M., Baranger, A., and Coyne, C.J. 2002. Quantitative trait loci for partial resistance to *Aphanomyces* root rot in pea. Theor. App. Genet. **106**(1): 28-39.

Pinzino, C., Capocchi, A., Galleschi, L., Saviozzi, F., Nanni, B., and Zandomeneghi, M. 1999. Aging, free radicals, and antioxidants in wheat seeds. J. Agric. Food Chem. **47**(4): 1333-1339.

Polle, A. 1997. Defense against photooxidative damage in plants. . *In* Oxidative Stress and the Molecular Biology of Antioxidant Defenses. *Edited by* J.G. Scandalios. Cold Spring Harbor Lab. Press New York.

Powell, W., Thomas, W.T.B., Baird, E., Lawrence, P., Booth, A., Harrower, B., McNicol, J.W., and Waugh, R. 1997. Analysis of quantitative traits in barley by the use of Amplified Fragment Length Polymorphisms. Heredity, **79**(1): 48-59.

Prasil, O., Adir, N., and Ohad, I. 1992. Dynamics of photosystem II: mechanism of photoinhibition and recovery process. *In* Topics in photosynthesis, the photosystems:structure, function and molecular biology, *Edited by* Barber, J. Vol 11. Elsevier, Amsterdam. pp. 220-250.

Prioul, S., Frankewitz, A., Deniot, G., Morin, G., and Baranger, A. 2004. Mapping of quantitative trait loci for partial resistance to *Mycosphaerella pinodes* in pea (*Pisum sativum* L.) at the seedling and adult plant stages. Theor. App. Genet. **108**(7): 1322-1334.

Quesnelle, P.E., and Emery, R.J.N. 2007. cis-Cytokinins that predominate in *Pisum sativum* during early embryogenesis will accelerate embryo growth in vitro. Can. J. Botany, **85**: 91-103.

Radchuk, R., Radchuk, V., Weschke, W., Borisjuk, L., and Weber, H. 2006. Repressing the Expression of the SUCROSE NONFERMENTING-1-RELATED PROTEIN KINASE gene in pea embryo causes pleiotropic defects of maturation similar to an abscisic acid-insensitive phenotype. Plant Physiol. **140**(1): 263-278.

Rajcan, I., Kasha, K.J., Kott, L.S., and Beversdorf, W.D. 2002. Evaluation of cytoplasmic effects on agronomic and seed quality traits in two doubled haploid populations of *Brassica napus* L. Euphytica, **123**(3): 401-409.

Raz, V., Bergervoet, J.H., and Koornneef, M. 2001. Sequential steps for developmental arrest in Arabidopsis seeds. Development, **128**(2): 243-252.

Riehle, W.G., and Muelbauer, F.J. 1975. Laboratory technique for bleaching pea seeds. Crop Sci **15**(6): 853-855.

Rock, C.D., and Quatrano, R.S. 1995. The role in hormones during seed development. *In* Plant hormones: Physiology, biochemistry and molecular biology. *Edited by* P.J. Davies. Kluwer Academic Publishers, Dordrecht, Netherlands. pp. 671-697.

Rolletschek, H., Weber, H., and Borisjuk, L. 2003. Energy status and its control on embryogenesis of legumes. Embryo photosynthesis contributes to oxygen supply and is coupled to biosynthetic fluxes. Plant Physiol. **132**(3): 1196-1206.

Roszak, A.W., McKendrick, K., Gardiner, A.T., Mitchell, I.A., Isaacs, N.W., Cogdell, R.J., Hashimoto, H., and Frank, H.A. 2004. Protein regulation of carotenoid binding: Gatekeeper and locking amino acid residues in reaction centers of *Rhodobacter sphaeroides*. Structure, **12**(5): 765-773.

Rowell, J.G., and Walters, D.E. 1976. Analyzing data with repeated observations on each experimental unit. J. Agric. Sci. **87**: 423-432.

Ruan, Y., Gilmore, J., and Conner, T. 1998. Towards Arabidopsis genome analysis: monitoring expression profiles of 1400 genes using cDNA microarrays. Plant J. **15**(6): 821-833.

Ruuska, S.A., Girke, T., Benning, C., and Ohlrogge, J.B. 2002. Contrapuntal networks of gene expression during Arabidopsis seed filling. Plant Cell, **14**: 1191-1206.

Saeed, A.L., Sharov, V., White, J.L., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavish, A., Trush, V., and Quackenbush, J. 2003. TM4: A free, open-source system for microarray data management and analysis. Biotechniques, **34**(2): 374-378.

Sambrook, J., and Russel, D.W. 2001. Molecular Cloning, Volume 3, 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sagan, M., Ney, B., and Duc, G. 1993. Plant symbiotic mutants as a tool to analyse nitrogen nutrition and yield relationship in field-growth peas (*Pisum sativum* L.). Plant Soil, **153**(1): 33-45.

Sagar, A.D., Horwitz, B.A., Elliott, R.C., Thompson, W.F., and Briggs, W.R. 1988. Light effects on several chloroplast components in norflurazon-treated pea seedlings. Plant Physiol. **88**(2): 340-347.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**(4839): 487-491.

Salas, P., Oyarzo-Llaipen, J., Wang, D., Chase, K., and Mansur, L. 2006. Genetic mapping of seed shape in three populations of recombinant inbred lines of soybean (*Glycine max* L. Merr.). Theor. App. Genet. **113**(8): 1459-1466.

Sandmann, G., Kuhn, M., and Böger, P. 1993. Carotenoids in photosynthesis: Protection of D1 degradation in the light. Phot. Synth. Res. **35**(2): 185-190.

SAS Institute Inc. 1997. SAS/STAT user's guide. SAS Institute Inc., Cary, NC. USA.

Sato, K., and Takeda, K. 2009. An application of high-throughput SNP genotyping for barley genome mapping and characterization of recombinant chromosome substitution lines. Theor. App. Genet. **119**(4): 613-619.

Sax, K. 1923. The association of size differences with seed-coat patterns and pigmentation in *Phaseolus vulgaris*. Genetics **8**(6): 552-560.

Schaffer, R., Landgraf, J., Perez-Amador, M., and Wisman, E. 2000. Monitoring genomi-wide expression in plants. Curr. Opin. Biotechnol. **11**(162-167).

Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science, **270**(5235): 467-470.

Schmalenbach, I., Körber, N., and Pillen, K. 2008. Selecting a set of wild barley introgression lines and verification of QTL effects for resistance to powdery mildew and leaf rust. Theor. App. Genet. **117**(7): 1093-1106.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. **18**(2): 233-234.

Shepherd, A.D. 1959. Effect of illumination on color of frozen peas packaged in a transparent film. Food Technol. **9**: 539-540.

Sims, D.A., and Gamon, J.A. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. Remote Sens. Environ. **81**(2-3): 337-354.

Slinkard, A.E., Silh, M.B., and Vandenberg, A. 2000. Breeding for yield: The direct approch. *In* Linking research and marketing opportunities for pulses in the 21st Century. *Edited by* R. Knight. Kluwer Academic Publishers, Netherlands. pp. 183-190.

Smith, D.L. 1973. Nucleic acid, protein and starch synthesis in developing cotyledons of *Pisum arvense* L. Ann. Botany, **37**: 795-804.

Snoad, B., and Arthur, A.E. 1973. Genetical studies of quantitative characters in peas. 2. A six-parent diallel cross of cultivars and primitive forms. Euphytica **22**(3): 510-519.

Somers, D., Isaac, P., and Edwards, K. 2004. A high-density microsatellite consensus map for bread wheat (*Triticum aestivum* L.). Theor. App. Genet. **109**(6): 1105-1114.

Son, L.V., Tiedemann, J., Rutten, T., Hiller, S., Hinz, G., Zank, T., Manteuffel, R., and Baumlein, H. 2009. The BURP domain protein AtUSPL1 of *Arabidopsis thaliana* is destined to the protein storage vacuoles and over expression of the cognate gene distorts seed development. Plant Mol. Biol. **71**: 319 - 329.

Statistics Canada, 2009. Cereals and Oilseeds Review. [on line]. Catalogue No. 22-007-X. Vol 32(5). Statistics Canada. Ottawa, Ont. Available from http://dsp-psd.pwgsc.gc.ca/collection_2009/statcan/22-007-X/22-007-x2009005-eng.pdf [accessed 1 August 2009].

Steet, J.A., and Tong, C.H. 1996. Degradation kinetics of green color and chlorophylls in peas by colorimetry and HPLC. J. Food Sci. **61**(5): 924-928.

Steiger, S., Schäfer, L., and Sandmann, G. 1999. High-light-dependent up-regulation of carotenoids and their antioxidative properties in the *Syanobacterium synechocystis* PCC 6803. J. Photoc. Photobio. B. **52**(1-3): 14-18.

Swiecicki, W.K., and Timmerman-Vaughan, G. 2005. Localization of important traits: The example pea (*Pisum sativum* L.). *In* Molecular Marker Systems in Plant Breeding and Crop Improvement. pp. 155-169.

Tanksley, S.D. 1993. Mapping Polygenes. Ann. Rev. Genet. **27**(1): 205-233.

Tar'an, B., Warkentin, T., Somers, D.J., Miranda, D., Vandenberg, A., Blade, S., and Bing, D. 2004. Identification of quantitative trait loci for grain yield, seed protein concentration and maturity in field pea (*Pisum sativum* L.). Euphytica **136**(3): 297-306.

Tar'an, B., Warkentin, T., Somers, D.J., Miranda, D., Vandenberg, A., Blade, S., Woods, S., Bing, D., Xue, A., DeKoeyer, D., and Penner, G. 2003. Quantitative trait loci for lodging resistance, plant height and partial resistance to mycosphaerella blight in field pea (*Pisum sativum* L.). Theor. App. Genet. **107** (8): 1482-1491.

Tautz, D. 1989. Hypervariabflity of simple sequences as a general source for polymorphic DNA markers. Nucl. Acids Res. **17**(16): 6463-6471.

Telfer, A. 2002. What is β-carotene doing in the photosystem II reaction centre ? Philos. T. R. Soc. Lon. B. **357**: 1431-1440

Thoday, J.M. 1961. Location of polygenes. Nature, **9**: 368-370.

Timmerman, G.M., Frew, T.J., Miller, A.L., Weeden, N.F., and Jermyn, W.A. 1993. Linkage mapping of *sbm-1*, a gene conferring resistance to pea seed-borne mosaic virus, using molecular markers in (*Pisum sativum* L.). Theor. App. Genet. **85** (5): 609-615.

Timmerman, G.M., Frew, T.J., Weeden, N.F., Miller, A.L., and Goulden, D.S. 1994. Linkage analysis of *er-1*, a recessive (*Pisum sativum* L.); gene for resistance to powdery mildew fungus (*Erysiphe pisi* D.C.). Theor. App. Genet. **88**(8): 1050-1055.

Timmerman-Vaughan, G.M., Frew, T.J., Butler, R., Murray, S., Gilpin, M., Falloon, K., Johnston, P., Lakeman, M.B., Russell, A., and Khan, T. 2004. Validation of quantitative trait loci for Ascochyta blight resistance in pea (*Pisum sativum* L.), using populations from two crosses. TAG. Theor. App. Genet. **109**(8): 1620-1631.

Timmerman-Vaughan, G.M., Frew, T.J., Russell, A.C., Khan, T., Butler, R., Gilpin, M., Murray, S., and Falloon, K. 2002. QTL Mapping of partial resistance to field epidemics of *Ascochyta* Blight of Pea. Crop Sci. **42**(6): 2100-2111.

Timmerman-Vaughan, G.M., McCallum, J.A., Frew, T.J., Weeden, N.F., and Russell, A.C. 1996. Linkage mapping of quantitative trait loci controlling seed weight in pea (*Pisum sativum* L.). Theor. App. Genet. **93**(3): 431-439.

Trebst, A. 2002. A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintainance of photosystem II structure and function in *Clamydomonas reinhardii*. FEBS Letters, **516**: 156-160

Ubayasena, L., Bett, K., Tar'an, B., Perumal, V., and Warkentin, T. 2010. Genetic control and QTL analysis of cotyledon bleaching resistance in green field pea (*Pisum sativum* L.). Genome, **53**(5): 346-359.

Valderrama, M.R., Román, B., Satovic, Z., Rubiales, D., Cubero, J.I., and Torres, A.M. 2004. Locating quantitative trait loci associated with *Orobanche crenata* resistance in pea. Weed Res. **44**(4): 323-328.

Van Dongen, J.T., Ammerlaan, A.M.H., Wouterlood, M., Van Aelst, A.C., and Borstlap, A.C. 2003. Structure of the developing pea seed coat and the post-phloem transport pathway of nutrients. Ann. Bot. **91**(6): 729-737.

Van Ooijen, J.W. (ed.) 2004. MapQTL® 5, Software for the mapping of quantitative traits loci in experimental populations. . Kyazma B.V., Wageningen, Netherlands.

Van Ooijen, J.W. 1992. Accuracy of mapping quantitative traits loci in autogamous species. Theor. App. Genet. **84**: 803-811.

Van Ooijen, J.W., and Voorips, R.E. 2004. JoinMap Version 3.0, Software for the calculation of genetic linkage maps. Kyazma B.V., Wageningen, Netherlands.

van Sanford, D.A., and Matzinger, D.F. 1982. Dirrect and maternal variances of seedling characters in tobacco. . Crop Sci. **22**: 1213-1218.

Velculescu, V.E., Zhang, L., Vogelstein, B., and Kinzler, K.W. 1995. Serial analysis of gene expression. Science, **270**(5235): 484-487.

Venter, M., and Botha, F.C. 2004. Promoter analysis and transcription profiling: Integration of genetic data enhances understanding of gene expression. Physiol. Plantarum **120**(1): 74-83.

Visscher, P.M., Thompson, R., and Haley, C.S. 1996. Confidence intervals in QTL mapping by bootstrapping. Genetics, **143**(2): 1013-1020.

von Stackelberg, M., Lindemann, S., Menke, M., Riesselmann, S., and Jacobsen, H.J. 2003. Identification of AFLP and STS markers closely linked to the def locus in pea. Theor. App. Genet. **106**(7): 1293-1299.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T.v.d., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. **23**(21): 4407-4414.

Wang, A., Xia, Q., Xie, W., Datla, R., and Selvaraj, G. 2003. The classical Ubisch bodies carry a sporophytically produced structural protein (PAFTIN) that is essential for pollen development. Proc. Natl. Acad. Sci. USA **100**: 14487 - 14492.

Wang, T.L., Hadavizideh, A., Harwood, A., Welham, T.J., Harwood, W.A., Faulks, R., and Hedley, C.L. 1990. An Analysis of Seed Development in *Pisum sativum*. XIII. The Chemical Induction of Storage Product Mutants. Plant Breeding, **105**(4): 311-320.

Warkentin, T., Vadenberg, A., Banniza, S., and Slinkard, A. 2005. CDC Bronco field pea. Can. J. Plant Sci. **85**(3): 649-650.

Warkentin, T., Vandenberg, A., Banniza, S., and Slinkard, A. 2004. CDC Striker field pea. Can. J. Plant Sci. **84**: 239-240.

Watson, C.F., Zheng, L., and DellaPenna, D. 1994. Reduction of tomato polygalacturonase beta subunit expression affects pectin solubilization and degradation during fruit ripening. Plant Cell, **6**: 1623 - 1634.

Weber, H., Borisjuk, L., and Wobus, U. 2005. Molecular physiology of legume seed development. Ann. Rev. Plant Biol. **56**(1): 253-279.

Weber, J.L., and May, P.E. 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. **44**: 388-396.

Weeden, N.F., and Marx, G.A. 1984. Chromosomal locations of twelve isozyme loci in *Pisum sativum*. J. Heredity, **75**(5): 365-370.

Weeden, N.F., and Marx, G.A. 1987. Further genetic analysis and linkage relationships of isozyme loci in the pea: Confirmation of the diploid nature of the genome. J. Hered. **78**(3): 153-159.

Weeden, N.F., and Wolko, B. 1990. *Pisum sativum* (Garden pea). *In* Genetic maps: Locus of complex genomes. *Edited by* S.J. O'Brien. Cold Spring Harbor Laboratory Press, New York. pp. 6.106-6.112.

Weeden, N.F., Swiecicki, W.K., Ambrose, M.J., and Timmerman, G.M. 1993. Linkage groups of pea. Pisum Genet. **25**: 4.

Weeden, N.F., Timmerman, G.M., Ellis, T.H.N., Swiecicki, W.K., Rosov, S., and Berdnikov, V. 1998. A consensus map of the pea genome. Pisum Genet. **30**: 1-4.

Weller, J. I. 1986. Maximum likelihood techniques for the mapping and analysis of quantitative trait loci with the aid of genetic markers. Biometrics, **42**(3): 627-640.

Wellmer, F., Riechmann, J.L., Alves-Ferreira, M.r., and Meyerowitz, E.M. 2004. Genome-wide analysis of spatial gene expression in Arabidopsis flowers. Plant Cell, **16**(5): 1314-1326.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. **18**(22): 6531-6535.

Wright, S. 1980. Genic and organismic selection. Evolution **34**: 825-843.

Xu, H., Yao, G., Xiong, L., Yang, L., Jiang, Y., Fu, B., Zhao, W., Zhang, Z., Zhang, C., and Ma, Z. 2008. Identification and mapping of *pm2026*: a recessive powdery mildew resistance gene in an einkorn (*Triticum monococcum* L.) accession. Theor. App. Genet. **117**(4): 471-477.

Yan, M., Liu, Z., Guan, C., Chen, S., Yuan, M., and Liu, X. 2009. Inheritance and molecular markers for the seed coat color in *Brassica juncea*. Frontiers Agric. China, **3**(1): 1-6.

Yang, X., Yan, J., Shah, T., Warburton, M., Li, Q., Li, L., Gao, Y., Chai, Y., Fu, Z., Zhou, Y., Xu, S., Bai, G., Meng, Y., Zheng, Y., and Li, J. 2010. Genetic analysis and characterization of a new maize association mapping panel for quantitative trait loci dissection. Theor. App. Genet. **121**(3): 417-431.

Yang, Y.H., and Speed, T. 2002. Design issues for cDNA microarray experiments. Nat. Rev. Genet. **3**: 579-588.

Young, N.D. 1996. QTL mapping and quantitative disease resistance in plants. Ann. Rev. Phytopathl. **34**(1): 479-501.

Yu, J., Gu, W.K., Provvidenti, R., and Weeden, N.F. 1995. Identifying and mapping two DNA markers linked to the gene conferring resistance to pea enation mosaic virus. J. Am. Soc. Hort. Sci. **120**(5): 730-733.

Zhang, W., Xu, H., and Zhu, J. 2009. Index selection on seed traits under direct, cytoplasmic and maternal effects in multiple environments. J. Genet. Genomics, **36**(1): 41-49.

Zheng, L., Heupel, R.C., and DellaPenna, D. 1992. The beta subunit of tomato fruit polygalacturonase isoenzyme 1: isolation, characterization, and identification of unique structural features. Plant Cell, **4**: 1147 - 1156.

Zheng, L., Watson, C.F., and DellaPenna, D. 1994. Differential expression of the two subunits of tomato polygalacturonase isoenzyme 1 in wild-type rin tomato fruit. Plant Physiol. **105**: 1189 - 1195.

Zonneveld, B.J.M., Leitch, I.J., and Bennett, M.D. 2005. First Nuclear DNA Amounts in more than 300 Angiosperms. Ann. Bot. **96**(2): 229-244.

APPENDICES

Appendix 1. Photographs illustrating the visual quality traits in pea investigated in this thesis.

A. Typical 'CDC Striker' sample showing bleaching resistance, round shape and no dimples
B. Typical 'CDC Bronco' sample showing bright yellow, round shape and no dimples
C. Green pea line showing slightly blocky shape and bleaching susceptibility
D. Green pea line showing blocky seed shape and slightly dimpled
E. Green pea line showing moderately blocky seed shape and severely dimpled
F. Typical 'Alfetta' sample showing blocky seed shape, slightly greenish and moderately dimpled
G. Typical 'Orb' sample showing blocky seed shape, slightly dimpled and severely bleached
H. Green pea line showing blocky seed shape, moderately dimpled and severely bleached

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Appendix 4. QTLs identified for lightness (WSL) and greenness (WSa) of yellow pea seeds based on the Alfetta X CDC Bronco population in two locations over two years, with assessment of whole seeds using the Hunter Lab colorimeter.

Phenotype	Year	Location	Linkage group	QTL region, location and LOD value			LOD[c]	r^2 (%)[d]	Add. Effect[e]
				Closest Marker[a]	Location (cM)	Maximum LOD[b]			
Lightness	2006	Rosthern	LG I	C19-308	0.0-6.2	5.7* (2.9)	5.7*	9.8	0.44 (Alfetta)
Lightness	2006	Rosthern	LG I	AGTC152	12.8-18.2	3.4* (2.9)	3.3*	5.3	0.34 (Alfetta)
Lightness	2006	Rosthern	LG II-2	AGTG202	16.0-23.8	3.0* (2.9)	3.0*	4.8	0.28 (CDC Bronco)
Lightness	2006	Rosthern	LG II-2	CAAT170	43.8-47.7	5.9* (2.9)	5.8*	10.1	0.42 (Alfetta)
Lightness	2006	Rosthern	LG VII-2	AGAT220	113.4-119.3	5.0* (2.9)	5.0*	8.5	0.41 (CDC Bronco)
Lightness	2006	Saskatoon	LG I	AA102-2_214	6.8-7.8	2.7* (2.5)	2.3*	7.1	0.58 (CDC Bronco)
Lightness	2006	Saskatoon	LG II-2	CAAT170	43.8-48.7	5.2* (2.5)	5.2*	17.3	0.56 (Alfetta)
Lightness	2007	Rosthern	LG I	CCTC200	0.0-2.0	6.1* (3.0)	6.1*	22.2	0.50 (CDC Bronco)
Lightness	2007	Saskatoon	LG VII-2	CAAA232	18.0	2.3* (2.2)	2.3*	10.4	0.49 (Alfetta)
Lightness	2007	Saskatoon	LG VII-2	CATG207	92.2-92.8	3.8* (2.2)	3.7*	18.8	-1.59 (Alfetta)
Greenness	2006	Rosthern	LG I	CCTT186	29.0	3.1* (3.0)	3.1*	11.3	0.20 (Alfetta)
Greenness	2006	Saskatoon	LG II-2	AGAC198	24.9-28.7	3.8* (2.9)	3.9*	15.3	-0.19 (Alfetta)
Greenness	2006	Saskatoon	LG I	AA102-2_214	6.8	2.9* (2.9)	2.9*	11.4	0.17 (CDC Bronco)

* = Significant QTL at P≤0.05 after 1000 permutations

[a] = Closest marker to the identified QTL with maximum LOD value

[b] = Values in parenthesis represent the threshold LOD value at P≤0.05 after 1000 permutations

[c] = LOD value of the closest marker

[d] = Percentage of total variability explained by the QTL detected for the trait

[e] = Additive effect for QTL detected and the responsible parent contributing to increase the value of the trait

Appendix 5. QTLs identified for seed shape as measured by the percentage of round seeds based on the Alfetta X CDC Bronco and Orb X CDC Striker populations in two locations over two years.

Population	Year	Location	Linkage group	QTL region, location and LOD value			LOD[c]	r^2 (%)[d]	Add. Effect[e]
				Closest Marker[a]	Location (cM)	Maximum LOD[b]			
Alfetta X CDC Bronco	2006	Rosthern	LG VII-2	AGTG138	33.5-36.7	5.3* (3.0)	5.3*	14.8	3.3 (CDC Bronco)
Alfetta X CDC Bronco	2006	Rosthern	LG I	C19-352	9.4-10.1	3.8* (3.0)	3.8*	10.4	-4.1 (Alfetta)
Alfetta X CDC Bronco	2006	Saskatoon	LG VII-2	CGAG219	64.7-66.7	3.7* (2.9)	3.7*	14.7	2.9 (CDC Bronco)
Alfetta X CDC Bronco	2006	Saskatoon	LG VII-1	CCTA168	58.5-59.5	3.3* (2.9)	2.9*	11.2	2.5 (Alfetta)
Alfetta X CDC Bronco	2007	Rosthern	LG VII-2	CAAC286	73.9-74.7	4.3* (2.9)	4.3*	17.4	2.4 (CDC Bronco)
Alfetta X CDC Bronco	2007	Rosthern	LG I	CCTG187	30.0-33.0	3.0* (2.9)	2.9*	11.2	-1.9 (CDC Bronco)
Alfetta X CDC Bronco	2007	Saskatoon	LG VII-2	CGAG219	62.7-71.7	6.0* (3.0)	5.7*	25.0	3.6 (CDC Bronco)
Orb X CDC Striker	2006	Rosthern	LG I-2	AGAC190	73.3-74.8	3.7* (3.1)	3.7*	9.3	-3.1 (Orb)
Orb X CDC Striker	2006	Rosthern	LG IV-2	CCAG207	63.4-71.1	6.4* (3.1)	6.4*	17.0	4.6 (Orb)
Orb X CDC Striker	2006	Saskatoon	LG IV-1	AGTC435	47.1-51.3	5.6* (2.9)	5.6*	23.4	6.8 (CDC Striker)
Orb X CDC Striker	2006	Saskatoon	LG IV-1	AGTC139	59.6	2.9* (2.9)	2.9*	11.5	-4.7 (Orb)
Orb X CDC Striker	2007	Rosthern	LG A	AGTA230	36.5-48.9	5.7* (3.1)	5.7*	14.2	2.5 (CDC Striker)
Orb X CDC Striker	2007	Rosthern	LG I-2	AA37-456	50.9-53.4	4.2* (3.1)	4.2*	10.3	-2.8 (CDC Striker)
Orb X CDC Striker	2007	Rosthern	LG IV-2	CCTA175	33.8-35.8	4.1* (3.1)	4.1*	10.1	4.6 (CDC Striker)
Orb X CDC Striker	2007	Rosthern	LG IV-2	ACAC105	49.6-51.0	3.8* (3.1)	3.8*	8.5	-3.2 (CDC Striker)
Orb X CDC Striker	2007	Rosthern	LG IV-2	CCAG207	69.0	3.1* (3.1)	3.1*	6.5	2.2 (Orb)
Orb X CDC Striker	2007	Saskatoon	LG I-2	AGTG295	63.9-66.2	3.4* (3.0)	3.2*	12.6	-3.0 (CDC Striker)
Orb X CDC Striker	2007	Saskatoon	LG A	AGTA230	38.5-41.5	3.9* (3.0)	2.5*	9.6	2.5 (CDC Striker)

* = Significant QTL at P≤0.05 after 1000 permutations
[a] = Closest marker to the identified QTL with maximum LOD value
[b] = Values in parenthesis represent the threshold LOD value at P≤0.05 after 1000 permutations
[c] = LOD value of the closest marker
[d] = Percentage of total variability explained by the QTL detected for the trait
[e] = Additive effect for QTL detected and the responsible parent contributing to increase the value of the trait

Appendix 6. QTLs identified for seed dimpling based on the Alfetta X CDC Bronco and Orb X CDC Striker populations, respectively, in two locations over two years.

Population	Year	Location		QTL region, location and LOD value			LOD[c]	r^2 (%)[d]	Add. Effect[e]
			Linkage group	Closest Marker[a]	Location (cM)	Maximum LOD[b]			
Alfetta X CDC Bronco	2006	Saskatoon	LG VII-2	AGTG138	33.5-36.7	4.9* (2.9)	4.4*	19.5	0.6 (CDC Bronco)
Alfetta X CDC Bronco	2007	Rosthern	LG VII-2	AGTG138	34.5-35.7	3.1* (2.9)	3.1*	13.2	0.4 (CDC Bronco)
Alfetta X CDC Bronco	2007	Saskatoon	LG VII-2	AGTG138	33.5-36.7	2.7^{NS} (2.9)	2.4^{NS}	10.8	0.3 (CDC Bronco)
Orb X CDC Striker	2006	Rosthern	LG IV-1	B17-320	0.0-1.0	3.5* (3.1)	3.5*	8.8	-0.2 (Orb)
Orb X CDC Striker	2006	Rosthern	LG IV-1	CGAA235	41.4	3.3* (3.1)	3.3*	8.2	0.6 (Orb)
Orb X CDC Striker	2006	Rosthern	LG IV-1	AGTC139	59.6	5.9* (3.1)	5.9*	15.6	0.8 (Orb)
Orb X CDC Striker	2006	Saskatoon	LG A	ACTC186	12.7-15.1	3.1* (3.0)	2.9*	14.0	-0.3 (Orb)
Orb X CDC Striker	2007	Rosthern	LG I-2	CCTC325	38.5-42.6	5.3* (3.0)	5.2*	16.2	- 0.5(Orb)
Orb X CDC Striker	2007	Rosthern	LG I-2	CCAC246	56.9-57.5	4.2* (3.0)	4.5*	13.1	0.7 (CDC Striker)
Orb X CDC Striker	2007	Saskatoon	LG IV-1	CCAG291	78.2-84.5	5.0* (3.1)	4.9*	20.3	0.4 (Orb)
Orb X CDC Striker	2007	Saskatoon	LG D	AGTA178	0.0-7.0	3.6* (3.1)	3.3*	12.9	0.3 (Orb)

§=Refer to Fig. 2 and 3

^{NS} = Not significant QTL at $P \leq 0.05$ after 1000 permutations

[a] = Closest marker to the identified QTL with maximum LOD value

[b] = Values in parenthesis represent the threshold LOD value at $P \leq 0.05$ after 1000 permutations

[c] = LOD value of the closest marker

[d] = Percentage of total variability explained by the QTL detected for the trait

[e] = Additive effect for QTL detected and the responsible parent contributing to increase the value of the trait

Appendix 7. List of differentially expressed genes of CDC Striker compared to Orb seed coats at 14 days after flowering (DAF)

ID	DAF	Annotation	p-value	M*	Function
14 DAF up-regulated genes in CDC Striker seed coats compaired to Orb (47)					
Maturation Growth & Development (14)					
PSOLI01214	14, 28	GPI-anchored protein	0.001	1.01	Anchored to membrane
PSOLI05000	14	Dormancy-associated protein (DRM1)	0.000	1.03	Maturation
PSOLI04214	14	Oleosin 3	0.000	1.10	Oil Body Membrane Protein
PSOLI04158	14	Seed maturation protein PM34	0.000	1.17	Oxidoreductase
PSOLI02729	14, 21	Lustrin A-like	0.000	1.34	Biomineralization proteins
PSOLI03086	14, 21	Senescence-associated protein-related	0.000	1.37	Senescence-associated protein-related
PSOLI04150	14	Oleosin 1	0.000	1.44	Integral to membrane
PSOLI02815	14, 21, 28	BURP domain containing protein	0.000	1.47	Seed development
PSOLI01681	14	Ntdin	0.000	1.48	Senescence-associated protein (SEN1)
PSOLI05018	14	Arabinogalactan-protein	0.000	1.54	Cell differentiation, cell-cell recognition, embryogenesis and programmed cell death
PSOLI02904	14	Serine-type endopeptidase activity	0.000	1.79	Proteolysis
PSOLI04532	14	Glycinin subunit	0.000	1.96	Seed storage protein
PSOLI01360	14	PsbP protein	0.000	1.98	Photosynthesis
PSOLI04441	14	Albumin 2	0.000	3.21	Seed storage protein
Metabolism (11)					
PSOLI01493	14	UDP-glucose glucosyltransferase	0.000	1.00	Glycosyltransferase
PSOLI03211	14, 21	Probable calcium-binding protein CML50	0.000	1.06	Calcium ion binding
PSOLI04409	14	Asparagine synthase 1	0.000	1.09	Asn synthesis
PSOLI04822	14	Galactinol synthase	0.000	1.12	Transferase activity
PSOLI00895	14, 21	Thioredoxin-like 3-2, chloroplastic	0.000	1.16	Probable thiol-disulfide oxidoreductase that may participate in various redox reactions
PSOLI00518	14	Glutamine synthetase	0.001	1.25	Nitrogen metabolisum
PSOLI02989	14	Glutamine synthetase	0.000	1.30	Glutamine biosynthetic process
PSOLI00729	14, 21	Alpha-amylase	0.000	1.40	Starch and sucrose metabolism
PSOLI03631	14	UDP-glucose glucosyltransferase	0.000	1.56	Transferase activity, transferring hexosyl groups
PSOLI04425	14, 21	Triosephosphate isomerase	0.000	1.65	Acyltransferase activity
PSOLI00638	14	Trypsin inhibitor protein	0.000	2.59	Serine-type endopeptidase inhibitor activity
Signaling (1)					
PSOLI04890	14, 21	1-aminocyclopropane-1-carboxylate oxidase	0.000	1.08	Ethylene biosynthesis
Stress & pathogen related (3)					
PSOLI03914	14	Cyanogenic beta-glucosidase	0.000	1.16	Hydrolyzes cyanoglucosides
PSOLI01645	14	Pre-mRNA-splicing factor SPF27 homolog	0.000	1.20	Enhanced susceptibility to virulent and avirulent pathogens
PSOLI04233	14	12-oxophytodienoate reductase (OPR2)	0.000	1.55	Involved in jasmonic acid biosynthesis
Transcription & Translation (5)					
PSOLI00449	14	40S ribosomal protein	0.000	1.13	Translation
PSOLI03860	14, 21	Histone H1 (PsH1b)	0.000	1.22	Histone
PSOLI04324	14	Ribosomal protein	0.000	1.63	Translation
PSOLI04535	14	Ribosomal protein L32	0.000	1.81	Translation
PSOLI03676	14	Probable small nuclear ribonucleoprotein F			A processing

Appendix 7. Continued

ID	DAF	Annotation	p-value	M*	Function
Transport & Protein processing (8)					
PSOLI04325	14	Tonoplast intrinsic protein alpha	0.000	1.05	Channel protein in tonoplast
PSOLI04842	14	phloem specific protein [Vicia faba]	0.000	1.05	Vesicle trafficking
PSOLI04738	14	Signal recognition particle 54 kDa subunit	0.000	1.10	SRP-dependent cotranslational protein targeting to membrane
PSOLI05036	14, 21	SHOOT1 protein	0.000	1.10	Protein binding
PSOLI04949	14	Oligopeptidase B	0.000	1.16	Serine-type endopeptidase activity
PSOLI03048	14	Bimodular protein	0.001	1.40	Lipid transport
PSOLI05022	14	Protein required for the fusion of transport vesicles	0.000	1.58	Protein transport
PSOLI00636	14	P54 protein; partial	0.000	1.95	Protein mobilization
Unknown (5)					
PSOLI02874	14, 21	Hypothetical protein predicted by Glimmer/Critica	0.000	1.02	Unknown
PSOLI00836	14	Protein of unknown function	0.000	1.05	Unknown
PSOLI01906	14	Putative secreted protein	0.000	1.42	Protein of unknown function
PSOLI01073	14, 21	Unknown protein	0.000	1.97	Unknown
PSOLI03711	14, 21, 28	Putative calreticulin protein	0.000	2.48	Unknown
14 DAF down-regulated genes in CDC Striker seed coats compaired to Orb (49)					
Maturation Growth & Development (6)					
PSOLI01280	14	Annexin (Annexin) Family	0.000	-3.12	Cell proliferation
PSOLI03272	14	Ferritin	0.000	-1.89	Fe storage
PSOLI01245	14	Ferritin 3, plast. Cowpea	0.000	-1.82	Fe strorage
PSOLI04742	14	Putative BURP domain containing protein	0.000	-1.56	Dehydration-responsive protein RD22 precursor
PSOLI04829	14	Pisum sativum mRNA for putative glycine rich protein	0.000	-1.34	Cell defferenciation
PSOLI05011	14	Starch branching enzyme	0.000	-1.04	Starch modification
Metabolism (15)					
PCPS17	14	Sucrose synthase 1	0.000	-3.44	Sucrose cleavage
PSOLI03262	14	Xyloglucan endo-transglycosylase	0.000	-2.16	Hydrolase activity, hydrolyzing O-glycosyl compounds
PSOLI00658	14	beta-amyrin synthase	0.000	-2.07	Intramolecular transferase activity
PSOLI01489	14	Pollen-specific protein precursor like	0.000	-1.94	Multi-copper oxidase
PSOLI00981	14, 21	Not56-like protein	0.000	-1.78	Protein amino acid glycosylation
PSOLI00894	14, 21, 28	Tetrapyrrole-binding protein	0.000	-1.65	Chloroplast precursor
PSOLI02900	14	Pisum sativum ribulose-1;5 bisphosphate carboxylas	0.000	-1.44	Phtosynthesis
PSOLI03615	14	Beta-galactosidase	0.000	-1.27	Carbohydrate metabolic process
PSOLI00349	14	Trypsin protein inhibitor 1	0.000	-1.26	Endopeptidase inhibitor activity
PSOLI00625	14, 21	Lectin-like receptor kinase 7	0.000	-1.24	Protein amino acid phosphorylation
PSOLI03095	14, 21	Phosphoglyceromutase	0.000	-1.23	Glycolysis
PSOLI02712	14	Caffeic acid 3-O-methyltransferase 1	0.000	-1.22	Aromatic compound metabolism; phenylpropanoid biosynthesis
PSOLI00421	14	ORNITHINE CARBAMOYLTRANSFERASE	0.000	-1.16	Amino acid biosynthesis and salvage
PSOLI04885	14	RuBisCO subunit	0.000	-1.11	Phtosynthesis
PSOLI04820	14, 21	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	0.000	-1.03	Glycolysis

Appendix 7. Continued

ID	DAF	Annotation	p-value	M*	Function
Signaling (2)					
PSOLI03900	14	Olfactory receptor mor231-4	0.001	-1.42	Olfactory receptor activity
PSOLI00813	14, 21, 28	Jasmonate ZIM domain-containing protein 3	0.001	-1.35	Repressor of jasmonate responses
Stress & pathogen related (6)					
PSOLI02641	14	Monocopper oxidase precursor	0.000	-2.18	Disease resistance
PSOLI05212	14	BURP domain-containing protein	0.000	-1.61	Putative dehydration-responsive /Resistant specific protein
PSOLI05217	14	BURP domain-containing protein	0.000	-1.13	Putative dehydration-responsive /Resistant specific protein
PSOLI05211	14	BURP domain-containing protein	0.000	-1.12	Putative dehydration-responsive /Resistant specific protein
PSOLI00589	14	Abscisic acid and environmental stress inducible protein	0.000	-1.12	Stress tolarance
PSOLI02389	14	Disease resistance res ponse/ dirigent-like protein	0.000	-1.10	Disease resistance
Transcription & Translation (3)					
PSOLI04308	14	La protein homolog (La ribonucleoprotein)	0.000	-3.22	May be involved in transcription termination
PSOLI04058	14	AT4g05020/T32N4_4	0.000	-1.43	avin adenine dinucleotide binding
PSOLI04064	14	rRNA 2'-O-methyltransferase fibrillarin 1	0.000	-1.03	Translation
Transport & Protein processing (7)					
PSOLI00800	14, 21	Probable aquaporin NIP5.1/Nodulin26-like major intrinsic protein	0.000	-1.69	Transport variety of uncharged solutes ranging from water to ammonia to glycerol
PSOLI03126	14	Cystatin-like protein	0.000	-1.32	Cysteine-type endopeptidase inhibitor activity
PSOLI05196	14	Putative chloroplast nucleoid DNA binding protein	0.001	-1.08	Proteolysis
PSOLI04024	14	Ribophorin II	0.000	-1.07	N-glycosylation of newly synthesised polypeptides
PSOLI02329	14	Lipid-transfer protein	0.000	-1.05	Lipid transport
PSOLI03246	14	Translocon-associated protein (TRAP)	0.000	-1.05	Membrane protein of the endoplasmic reticulum
PSOLI02345	14	Facilitated glucose transporter	0.000	-1.01	Cell surface adhesion protein
Unknown (10)					
PSOLI02068	14, 21	Hypothetical protein predicted by Glimmer/Critica	0.000	-2.34	Unknown
PSOLI05213	14	Hypothetical protein predicted by Glimmer/Critica	0.000	-1.44	Unknown
PSOLI05216	14	Cylicin-1	0.000	-1.31	Unknown
PSOLI02869	14, 21	Hypothetical protein predicted by Glimmer/Critica	0.000	-1.26	Unknown
PSOLI05215	14	Hypothetical protein predicted by Glimmer/Critica	0.000	-1.19	Unknown
PSOLI01244	14	Hypothetical protein predicted by Glimmer/Critica	0.000	-1.17	Unknown
PSOLI01886	14	BURP domain-containing protein 4	0.000	-1.14	Unknown
PSOLI05214	14, 28	Hypothetical protein predicted by Glimmer/Critica	0.000	-1.11	Unknown
PSOLI02961	14	Suspensor-specific protein	0.000	-1.02	Unknown
PSOLI04891	14	Expressed protein	0.000	-1.02	Unknown
M*: The mean centered relative gene expression value (in $\log_2$ scale)					

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Appendix 8. List of differentially expressed genes of CDC Striker compared to Orb seed coats at 21 days after flowering (DAF)

ID	DAF	Annotation	p-value	M*	Function
21 DAF up-regulated genes in CDC Striker seed coats compaired to Orb (28)					
Maturation Growth & Development (6)					
PSOLI03086	21, 14	Senescence-associated protein-related	0.000	1.14	Senescence-associated protein-related
PSOLI02729	21, 14	Lustrin A-like	0.000	1.17	Biomineralization proteins
PSOLI05087	21	Pea 1-aminocyclopropane-1-carboxylate oxidase	0.001	1.39	Oxidation of ACC to ethylene via N-hydroxyl-ACC
PSOLI04306	21	Annexin-related protein	0.000	1.82	Cell proliferation
PSOLI02815	21, 14, 28	BURP domain containing protein	0.000	1.87	Seed development
Metabolism (7)					
PSOLI00094	21	P.sativum 16S rRNA & tRNA-Val chloroplast genes	0.000	1.00	Phtosynthesis
PSOLI04805	21	UDP-D-xylose 4-epimerase	0.000	1.09	Carbohydrate metabolism
PSOLI03390	21	Glucose-6-phosphate/phosphate translocator 1	0.001	1.14	Phtosynthesis
PSOLI04425	21, 14	Triosephosphate isomerase	0.000	1.19	acyltransferase activity
PSOLI00729	21, 14	Alpha-amylase	0.000	1.24	Starch and sucrose metabolism
PSOLI03211	21, 14	Probable calcium-binding protein CML50	0.000	1.29	Calcium ion binding
PSOLI00895	21, 14	Thioredoxin-like 3-2, chloroplastic	0.000	1.88	Probable thiol-disulfide oxidoreductase, participate in various redox reactions
Signaling (3)					
PSOLI03553	21	X-linked retinitis pigmentosa GTPase regulator-protein 1	0.000	1.27	Sensory transduction
PSOLI04890	21, 14	1-aminocyclopropane-1-carboxylate oxidase	0.000	1.34	Ethylene biosynthesis
PSOLI03886	21	Probable calcium-binding protein CML50	0.000	1.37	Potential calcium sensor
Stress & pathogen related (1)					
PSOLI02983	21	Leucine-rich repeat transmembrane protein kinase	0.000	1.50	Pathogen response
Transcription & Translation (3)					
PSOLI03772	21	L.luteus genes for 5S and 18S ribosomal RNA	0.000	1.15	Transcription related
PSOLI02655	21	F7H2.15 protein	0.000	1.46	Ribosomal protein
PSOLI03860	21, 14	Histone H1 (PsH1b)	0.000	1.57	Histone
Transport & Protein processing (3)					
PSOLI05036	21, 14	SHOOT1 protein	0.000	1.09	Protein binding
PSOLI00768	21	Inorganic phosphate transporter 1-5	0.000	1.17	Vascuolar transport
PSOLI01563	21	Protein YIPF5 (YIP1 family member 5).	0.000	1.23	Membrane transport
Unknown (6)					
PSOLI00951	21, 28	Hypothetical protein predicted by Glimmer/Critica	0.000	1.15	Unknown
PSOLI03846	21	Protein of unknown function	0.000	1.22	Unknown
PSOLI02874	21, 14	Hypothetical protein predicted by Glimmer/Critica	0.000	1.78	Unknown
PSOLI00850	21	Hypothetical protein predicted by Glimmer/Critica	0.001	2.21	Unknown
PSOLI01073	21, 14	Unknown protein	0.000	2.37	Unknown
PSOLI03711	21, 14, 28	Putative calreticulin protein	0.000	3.31	Unknown

Appendix 8. Continued

ID	DAF	Annotation	p-value	M*	Function
21 DAF down-regulated genes in CDC Striker seed coats compaired to Orb (26)					
Maturation Growth and Development (3)					
PSOLI04536	21	Seed protein precursor	0.000	-1.79	Dehydration-induced protein RD22
PSOLI03966	21	Tubulin alpha-2/alpha-4 chain	0.000	-1.22	Cell proliferation
PSOLI04552	21	Cysteine proteinase	0.000	-1.10	Cysteine-type endopeptidase activity, Degradation of staorage priteins
Metabolism (12)					
PSOLI00981	21, 14	Not56-like protein	0.000	-3.55	Protein amino acid glycosylation
PSOLI00894	21, 14, 28	Tetrapyrrole-binding protein	0.000	-3.33	Chloroplast precursor
PSOLI02775	21	Enoyl CoA hydratase-like protein	0.000	-1.82	Fatty acid metabolism
PSOLI05008	21	Chlorophyll A-B binding protein	0.000	-1.61	Phtosynthesis
PSOLI00625	21, 14	Lectin-like receptor kinase 7	0.000	-1.53	Protein amino acid phosphorylation
PSOLI05007	21	Chlorophyll A-B binding protein	0.000	-1.36	Phtosynthesis
PSOLI03652	21	AT5g38200/MXA21_90	0.000	-1.32	Glutamine metabolic process
PSOLI02497	21	UDP-glucose 6-dehydrogenase	0.000	-1.32	Respiration
PSOLI00939	21	3-ketoacyl-CoA synthase 5	0.000	-1.26	Lipid metabolism
PSOLI03095	21, 14	Phosphoglyceromutase	0.000	-1.24	Glycolysis
PSOLI04820	21, 14	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	0.000	-1.13	Glycolysis
PSOLI04478	21, 28	ATP sulfurylase	0.000	-1.08	ATP sulfurylase and APS kinase activity
Signaling (1)					
PSOLI00813	21, 14, 28	Jasmonate ZIM domain-containing protein 3	0.000	-1.60	Repressor of jasmonate responses
Stress & pathogen related (2)					
PSOLI00404	21	Kunitz trypsin inhibitor M t.	0.000	-1.01	Pathogen response
PSOLI02395	21	Ninja-family protein AFP3	0.000	-1.00	Negative regulator of abscisic acid (ABA)
Transcription & Translation (3)					
PSOLI03332	21, 28	Eukaryotic translation initiation factor 3 subunit 4	0.000	-2.36	Translation
PSOLI02869	21, 14	Genome polyprotein	0.000	-1.31	RNA binding
PSOLI01899	21	Histone H2B	0.000	-1.14	Transcription related
Transport & Protein processing (3)					
PSOLI00800	21, 14	Nodulin26-like major intrinsic protein	0.000	-2.13	Transport variety of uncharged solutes ranging from water to ammonia to glycerol
PSOLI02917	21	Probable exocyst complex component 4	0.001	-1.63	Protein transport
PSOLI02276	21	Proton pyrophosphatase	0.000	-1.10	Vacuolar proton-pyrophosphatase
Unknown (2)					
PSOLI02068	21, 14	Hypothetical protein predicted by Glimmer/Critica	0.000	-3.12	Unknown
PSOLI01943	21	Hypothetical protein predicted by Glimmer/Critica	0.000	-1.09	Unknown

M*: The mean centered relative gene expression value (in $\log_2$ scale)

Appendix 9. List of differentially expressed genes of CDC Striker compared to Orb seed coats at 28 days after flowering (DAF)

ID	DAF	Annotation	p-value	M*	Function
28 DAF up-regulated genes in CDC Striker seed coats compared to Orb (20)					
Maturation Growth & Development (4)					
PSOLI01434	28	GAST-like gene product	0.001	1.06	Cell proliferation
PSOLI00776	28	C2 domain-containing protein	0.000	1.14	Cell membrane targeted proteins
PSOLI01214	28, 14	GPI-anchored protein	0.000	1.16	Anchored to membrane
PSOLI01995	28	Patellin-3	0.000	2.37	Cell cycle
Metabolism (7)					
PSOLI01846	28	3-ketoacyl-CoA syntheses 11	0.000	1.12	Lipid metabolism
PSOLI00621	28	Cystathionine-gamma-syntheses	0.001	1.13	Cellular amino acid metabolic process
PSOLI00578	28	Dihydrokaempferol 4-reductase family	0.000	1.21	Flavonoid biosynthesis.
PSOLI02696	28	Os05g0423500 protein	0.000	1.22	Protein amino acid phosphorylation
PSOLI02008	28	Fumarylacetoacetase	0.000	1.31	Aromatic amino acid family metabolic process
PSOLI02945	28	Quinone reductase	0.000	1.32	Respiration
PSOLI00436	28	Photosystem I assembly protein ycf3.	0.000	1.44	Photosynthesis
Stress & pathogen related (1)					
PSOLI03217	28	Thioredoxin-dependent peroxidase	0.001	1.72	Important in cell redox reactions
Transcription & Translation (4)					
PSOLI00301	28	Nonspecific lipid-transfer protein precursor	0.000	1.03	Wax and cutin deposition in the cell walls of expanding epidermal
PSOLI02097	28	60S ribosomal protein L21-2.	0.000	1.05	Transcription related
PSOLI01797	28	60S ribosomal protein L21-2.	0.000	1.19	Transcription related
PSOLI04482	28	Histone H4 variant TH091.	0.000	1.32	Transcription related
Unknown (4)					
PSOLI01683	28	Uncharacterized protein C19orf29	0.000	1.11	Unknown
PSOLI00951	28, 21	Hypothetical protein predicted by Glimmer/Critica	0.000	1.65	Unknown
PSOLI04567	28	Hypothetical protein predicted by Glimmer/Critica	0.000	1.66	Unknown
PSOLI03711	28, 21, 14	Putative calreticulin protein	0.000	3.19	Unknown

Appendix 9. Continued

ID	DAF	Annotation	p-value	M*	Function
28 DAF down-regulated genes in CDC Striker seed coats compared to Orb (44)					
Maturation Growth & Development (5)					
PSOLI05071	28	Lipoprotein	0.000	-2.29	Seed maturation protein
PSOLI04570	28	Seed maturation protein PM39	0.000	-1.79	Cellular protection
PSOLI02815	28, 21, 14	BURP domain containing protein	0.001	-1.70	Seed development
PSOLI05134	28	Vicia faba putative ABA-induced guard cell protein	0.000	-1.44	Maturation
PSOLI04967	28	Dormancy/Auxin related protein	0.000	-1.02	Maturation
Metabolism (16)					
PSOLI00894	28, 21, 14	Tetrapyrrole-binding protein	0.000	-4.64	Chloroplast precursor
PSOLI00009	28	Photosystem II thylakoid membrane protein	0.000	-2.07	Photosynthesis
PSOLI04977	28	Photosystem II protein D1	0.000	-2.00	Photosynthesis
PSOLI00525	28	Photosystem Q	0.000	-1.77	Photosynthesis
PSOLI01663	28	Chlorophyll a-b binding protein 8	0.000	-1.70	Photosynthesis, light harvesting
PSOLI02993	28	Plastid-lipid associated protein / fibrillin family protein	0.000	-1.63	Photosynthesis
PSOLI03998	28	Cinnamyl-alcohol dehydrogenase	0.000	-1.51	Cell wall metabolism
PSOLI04392	28	Auxin-induced beta-glucosidase	0.000	-1.48	CH-metabolism
PSOLI00974	28	Alpha-L-arabinofuranosidase	0.000	-1.40	Cell wall metabolism
PSOLI04604	28	12-oxophytodienoate reductase (OPR1)	0.000	-1.32	Jasmonic acid pathway
PSOLI04478	28, 21	ATP sulfurylase	0.001	-1.26	ATP sulfurylase and APS kinase activity
PSOLI04933	28	Pyridoxal-phosphate-dependent enzyme family protein	0.000	-1.22	Amino acid metabolism
PSOLI00529	28	VITAMIN C DEFECTIVE 2	0.000	-1.20	Ascorbate biosynthesis
PSOLI02756	28	Invertase/pectin methylesterase inhibitor family protein	0.000	-1.15	CH-metabolism
PSOLI03853	28	Gibberellin 2-beta-dioxygenase 8	0.001	-1.06	Diterpenoid biosynthesis.
PSOLI03766	28	Adenosylmethionine decarboxylase family protein	0.000	-1.04	Methylation cycle
Signaling (2)					
PSOLI00813	28, 21, 14	Jasmonate ZIM domain-containing protein 3	0.000	-1.72	Repressor of jasmonate responses
PSOLI04543	28	Light-regulated protein precursor.	0.000	-1.55	Expression is controlled by light
Stress & pathogen related (8)					
PSOLI04608	28	Epoxide hydrolase; putative	0.000	-2.11	Detoxification
PSOLI04798	28	Plastid-lipid associated protein / fibrillin family protein	0.000	-2.06	Photoprotection
PSOLI03402	28	Kunitz trypsin inhibitor M t.	0.000	-2.05	Pathogen response
PSOLI04970	28	Late Embryogenesis Abundant Proteins	0.000	-1.33	Desiccation Tolerance
PSOLI03837	28	Dehydrin-cognate	0.000	-1.17	Dehydration response protein
PSOLI00653	28	Dehydrin-cognate	0.000	-1.07	Dehydration response protein
PSOLI02772	28	MtN19-like protein	0.000	-1.05	Detoxification
PSOLI03005	28	Dehydrin-cognate	0.000	-1.02	Dehydration response protein

Appendix 9. Continued

ID	DAF	Annotation	p-value	M*	Function
Transcription & Translation (11)					
PSOLI00405	28	CAGL2	0.000	-2.45	Transcription related
PSOLI03332	28, 21	Eukaryotic translation initiation factor 3 subunit 4	0.000	-2.13	Transcription related
PSOLI01060	28	DR2 protein	0.000	-1.78	Transcription related
PSOLI04036	28	ARIADNE-like protein	0.000	-1.33	Nucleic acid/protein binding
PSOLI04677	28	Invertase inhibitor	0.000	-1.31	Control of sucrose transportation
PSOLI03474	28	Heat shock protein binding	0.000	-1.17	Protein binding
PSOLI02133	28	Patellin-5	0.001	-1.15	Membrane-trafficking
PSOLI04669	28	Zinc finger (CCCH-type) family protein	0.000	-1.13	Transcription activator
PSOLI04408	28	Coatomer, Epsilon1-COP	0.001	-1.07	Vesicle trafficking
PSOLI03135	28	Probable small nuclear ribonucleoprotein G	0.001	-1.03	mRNA processing
PSOLI03942	28	Urate oxidase	0.000	-1.03	Purine metabolism
Unknown (2)					
PSOLI05214	28, 14	Hypothetical protein predicted by Glimmer/Critica	0.001	-1.23	Unknown
PSOLI04875	28	Om(1E) protein	0.000	-1.07	Unknown
M*: The mean centered relative gene expression value (in $\log_2$ scale)					

Appendix 10. List of genes grouped into 11 sub clusters identified based on the transcription profiles of CDC Striker and Orb seed coats at 14, 21, and 28 dayas after flowering (DAF)

Cluster	Annotation	Function	Functional group	Expression level (M*)		
				14 DAF	21 DAF	28 DAF
Cluster A						
PSOLI04570	Seed maturation protein PM39	Cellular protection	Maturation Growth & Development	-0.42	0.06	-1.79
PSOLI05071	Lipoprotein	Seed maturation protein	Maturation Growth & Development	-0.44	-0.10	-2.29
PSOLI00525	Photosystem Q	Photosynthesis	Metabolism	-0.32	0.21	-1.77
PSOLI00009	Photosystem II thylakoid membrane protein	Photosynthesis	Metabolism	-0.41	0.18	-2.07
PSOLI04977	Photosystem II protein D1	Photosynthesis	Metabolism	0.08	0.17	-2.00
PSOLI02993	Fibrillin family protein	Photosynthesis	Metabolism	0.31	-0.20	-1.63
PSOLI01663	Chlorophyll a-b binding protein 8	Photosynthesis, light harvesting	Metabolism	0.27	-0.10	-1.70
PSOLI04543	Light-regulated protein precursor.	Expression is controlled by light	Signaling	0.36	-0.45	-1.55
PSOLI03402	Kunitz trypsin inhibitor M t.	Pathogen response	Stress & pathogen related	0.12	-0.03	-2.05
PSOLI04798	Fibrillin family protein	Senescence related	Stress & pathogen related	0.67	-0.19	-2.06
PSOLI04608	Epoxide hydrolase; putative	Membrane structure	Stress & pathogen related	0.75	-0.04	-2.11
PSOLI00405	CAGL2	Transcription related	Transcription & Translation	-0.41	-0.62	-2.45
Cluster B						
PSOLI05018	Arabinogalactan-protein	Differentiation, embryogenesis and programmed cell death	Maturation Growth & Development	1.54	0.55	0.09
PSOLI02904	Serine-type endopeptidase activity	Proteolysis	Maturation Growth & Development	1.79	0.64	-0.03
PSOLI01360	PsbP protein	Photosynthesis	Maturation Growth & Development	1.98	0.65	-0.23
PSOLI03086	Senescence-associated protein-related	Senescence-associated protein-related	Maturation Growth & Development	1.37	1.14	-0.13
PSOLI02729	Lustrin A-like	Biomineralization proteins	Maturation Growth & Development	1.34	1.17	-0.15
PSOLI02815	BURP domain containing protein	Seed development	Maturation Growth & Development	1.47	1.87	-1.70
PSOLI04306	Annexin-related protein	Cell proliferation	Maturation Growth & Development	0.60	1.82	0.06
PSOLI05087	Pea 1-aminocyclopropane-1-carboxylate oxidase	Oxidation of ACC to ethylene via N-hydroxyl-ACC	Maturation Growth & Development	0.85	1.39	-0.22
PSOLI03631	UDP-glucose glucosyltransferase	Transferase activity, transferring hexosyl groups	Metabolism	1.56	0.50	0.23
PSOLI02989	Glutamine synthetase	Glutamine biosynthetic process	Metabolism	1.30	0.61	0.13
PSOLI00518	Glutamine synthetase	Nitrogen metabolism	Metabolism	1.25	0.87	-0.49
PSOLI04604	12-oxophytodienoate reductase (OPR1)	Jasmonic acid pathway (Senescence related)	Metabolism	1.72	0.85	-1.32
PSOLI00895	Thioredoxin-like 3-2, chloroplastic	Thiol-disulfide oxidoreductase in various redox reactions	Metabolism	1.16	1.88	0.18
PSOLI04890	1-aminocyclopropane-1-carboxylate oxidase	Ethylene biosynthesis	Signaling	1.08	1.34	-0.24
PSOLI04233	12-oxophytodienoate reductase (OPR2)	Involved in jasmonic acid biosynthesis	Stress & pathogen related	1.55	0.78	-0.95
PSOLI02983	leucine-rich repeat transmembrane protein kinase	Pathogen response	Stress & pathogen related	0.63	1.50	0.27
PSOLI02655	F7H2.15 protein	Ribosomal protein	Transcription & Translation	0.82	1.46	-0.38
PSOLI05036	SHOOT1 protein	Protein binding	Transport & Protein processing	1.10	1.09	0.06
PSOLI04949	Oligopeptidase B	Serine-type endopeptidase activity	Transport & Protein processing	1.16	1.01	-0.17
PSOLI00836	Protein of unknown function	Unknown	Unknown	1.05	1.02	-0.04
PSOLI02874	Hypothetical protein	Unknown	Unknown	1.02	1.78	-0.29

Appendix 10. Continued

Cluster	Annotation	Function	Functional group	Expression level (M*)		
				14 DAF	21 DAF	28 DAF
Cluster C						
PSOLI03211	Probable calcium-binding protein CML50	Calcium ion binding	Metabolism	1.06	1.29	0.66
PSOLI00729	Alpha-amylase	Starch and sucrose metabolism	Metabolism	1.40	1.24	0.62
PSOLI04425	Triosephosphate isomerase	Acyltransferase activity	Metabolism	1.65	1.19	0.48
PSOLI03886	Probable calcium-binding protein CML50	Potential calcium sensor	Signaling	0.94	1.37	0.99
PSOLI03860	Histone H1 (PsH1b)	Histone	Transcription & Translation	1.22	1.57	0.65
PSOLI04324	Ribosomal protein	Translation	Transcription & Translation	1.63	0.91	1.54
PSOLI03676	Probable small nuclear ribonucleoprotein F	mRNA processing	Transcription & Translation	2.15	0.93	1.63
PSOLI05022	Fusion protein of transport vesicles with the Golgi complex	Protein transport	Transport & Protein processing	1.58	1.24	1.48
PSOLI00850	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	1.93	2.21	0.36
PSOLI01073	Unknown protein	Unknown	Unknown	1.97	2.37	0.37
PSOLI03711	Putative calreticulin protein	Unknown	Unknown	2.48	3.31	3.19
Cluster D						
PSOLI00094	P.sativum 16S rRNA & tRNA-Val chloroplast genes	Photosynthesis	Metabolism	-0.02	1.00	-0.79
PSOLI03414	Alpha-fucosidase	Plant growth regulatotory	Signaling	-0.41	1.40	-0.43
PSOLI03772	L.luteus genes for 5S and 18S ribosomal RNA	Transcription related	Transcription & Translation	-0.42	1.15	-0.66
PSOLI00768	Inorganic phosphate transporter 1-5	Vascuolar transport	Transport & Protein processing	0.40	1.17	-0.40
PSOLI03846	Protein of unknown function	Unknown	Unknown	0.52	1.22	-0.76
Cluster E						
PSOLI04150	Oleosin 1	integral to membrane	Maturation Growth & Development	1.44	-0.53	-0.55
PSOLI04158	Seed maturation protein PM34	Oxidoreductase	Maturation Growth & Development	1.17	-0.59	-0.13
PSOLI04214	Oleosin 3	Oil Body Membrane Protein	Maturation Growth & Development	1.10	-0.65	0.25
PSOLI04532	Glycinin subunit	Seed storage protein	Maturation Growth & Development	1.96	-0.33	0.09
PSOLI04441	Albumin 2	Seed storage protein	Maturation Growth & Development	3.21	0.16	0.92
PSOLI04822	Galactinol synthase	Transferase activity	Metabolism	1.12	-0.19	-0.29
PSOLI00638	Trypsin inhibitor protein	Serine-type endopeptidase inhibitor activity	Metabolism	2.59	-0.84	0.38
PSOLI03914	Cyanogenic beta-glucosidase	Hydrolyzes cyanoglucosides	Stress & pathogen related	1.16	-0.08	-0.01
PSOLI04535	Ribosomal protein L32	Translation	Transcription & Translation	1.81	-0.61	0.38
PSOLI04325	Tonoplast intrinsic protein alpha	Channel protein in tonoplast	Transport & Protein processing	1.05	-0.53	0.19
PSOLI03048	Bimodular protein	Lipid transport	Transport & Protein processing	1.40	-0.69	0.08
PSOLI00636	P54 protein; partial	Protein mobilization	Transport & Protein processing	1.95	-0.34	-0.01
PSOLI01906	Putative secreted protein	Protein of unknown function	Unknown	1.42	-0.51	0.45

Appendix 10. Continued

Cluster	Annotation	Function	Functional group	Expression level (M*)		
				14 DAF	21 DAF	28 DAF
Cluster F						
PSOLI01995	Patellin-3	Cell cycle	Maturation Growth & Development	0.68	0.26	2.37
PSOLI00776	C2 domain-containing protein	Cell membrane targetted proteins	Maturation Growth & Development	-0.05	0.21	1.14
PSOLI02696	Os05g0423500 protein	Protein amino acid phosphorylation	Metabolism	0.29	0.90	1.22
PSOLI00621	Cystathionine-gamma-synthase	Cellular amino acid metabolic process	Metabolism	0.64	0.95	1.13
PSOLI02008	Fumarylacetoacetase	Aromatic amino acid family metabolic process	Metabolism	0.57	0.18	1.31
PSOLI01846	3-ketoacyl-CoA synthase 11	Lipid metabolism	Metabolism	0.06	0.21	1.12
PSOLI00578	Dihydroflavonol 4-reductase family	Flavonoid biosynthesis.	Metabolism	-0.07	0.05	1.21
PSOLI00436	Photosystem I assembly protein ycf3.	Photosynthesis	Metabolism	-0.35	0.49	1.44
PSOLI02945	Quinone reductase	Respiration	Metabolism	-0.19	-0.13	1.32
PSOLI01214	GPI-anchored protein	Anchored to membrane	Maturation Growth & Development	1.01	0.35	1.16
PSOLI03217	Thioredoxin-dependent peroxidase	Cell redox homeostasis	Stress & pathogen related	-0.18	-0.03	1.72
PSOLI02097	60S ribosomal protein L21-2.	Transcription related	Transcription & Translation	0.80	0.35	1.05
PSOLI01797	60S ribosomal protein L21-2.	Transcription related	Transcription & Translation	0.53	0.13	1.19
PSOLI04482	Histone H4 variant TH091.	Transcription related	Transcription & Translation	-0.06	-0.19	1.32
PSOLI00951	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	0.39	1.15	1.65
PSOLI04567	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	0.25	0.65	1.66
PSOLI01683	Uncharacterized protein C19orf29	Unknown	Unknown	0.25	0.56	1.11
Cluster G						
PSOLI05011	Starch branching enzyme	Starch modification	Maturation Growth & Development	-1.04	-0.08	-0.28
PSOLI02712	Caffeic acid 3-O-methyltransferase 1	Aromatic compound metabolism; phenylpropanoid biosynthesis	Metabolism	-1.22	-0.30	-0.23
PSOLI02900	Pisum sativum ribulose-1;5 bisphosphate carboxylas	Photosynthesis	Metabolism	-1.44	-0.17	0.36
PSOLI03615	Beta-galactosidase	Carbohydrate metabolic process	Metabolism	-1.27	-0.20	0.26
PSOLI04885	RuBisCO subunit	Photosynthesis	Metabolism	-1.11	-0.41	0.38
PSOLI03900	Olfactory receptor mor231-4	Olfactory receptor activity	Signaling	-1.42	-0.05	0.05
PSOLI05217	BURP domain-containing protein	Putative dehydration-responsive /Resistant specific protein	Stress & pathogen related	-1.13	0.08	-0.15
PSOLI04058	AT4g05020/T32N4_4	Avin adenine dinucleotide binding	Transcription & Translation	-1.43	-0.43	0.12
PSOLI04064	rRNA 2'-O-methyltransferase fibrillarin 1	Translation	Transcription & Translation	-1.03	-0.38	0.13
PSOLI03246	Translocon-associated protein (TRAP)	Membrane protein of the endoplasmic reticulum	Transport & Protein processing	-1.05	-0.01	-0.17
PSOLI02345	Facilitated glucose transporter	Cell surface adhesion protein	Transport & Protein processing	-1.01	-0.04	-0.06
PSOLI03126	Cystatin-like protein	Cysteine-type endopeptidase inhibitor activity	Transport & Protein processing	-1.32	0.03	-0.04

Appendix 10. Continued

Cluster	Annotation	Function	Functional group	Expression level (M*)		
				14 DAF	21 DAF	28 DAF
Cluster H						
PSOLI03272	Ferritin	Fe storage	Maturation Growth & Development	-1.89	-0.35	0.29
PSOLI01245	Ferritin 3, plast. Cowpea	Fe storage	Maturation Growth & Development	-1.82	-0.20	0.49
PSOLI01280	Annexin (Annexin) Family	Cell proliferation	Maturation Growth & Development	-3.12	-0.52	-0.49
PSOLI00658	Beta-amyrin synthase	Intramolecular transferase activity	Metabolism	-2.07	-0.43	0.06
PSOLI01489	Pollen-specific protein precursor like	Multi-copper oxidase	Metabolism	-1.94	-0.80	-0.16
PSOLI03262	Xyloglucan endo-transglycosylase	Hydrolase activity, hydrolyzing O-glycosyl compounds	Metabolism	-2.16	-0.06	-0.22
PCPS17	Sucrose synthase 1	Sucrose cleavage	Metabolism	-3.44	0.12	-0.57
PSOLI02641	Monocopper oxidase precursor	Disease resistance	Stress & pathogen related	-2.18	-0.67	0.25
PSOLI04308	La protein homolog (La ribonucleoprotein)	May be involved in transcription termination	Transcription & Translation	-3.22	-0.01	0.02
Cluster I						
PSOLI04536	Seed protein precursor	Dehydration-induced protein RD22	Maturation Growth & Development	-0.74	-1.79	-1.41
PSOLI00981	Not56-like protein	Protein amino acid glycosylation	Metabolism	-1.78	-3.55	-2.28
PSOLI00894	Tetrapyrrole-binding protein	Chloroplast precursor	Metabolism	-1.65	-3.33	-4.64
PSOLI00813	Jasmonate ZIM domain-containing protein 3	Repressor of jasmonate responses	Signaling	-1.35	-1.60	-1.72
PSOLI03332	Eukaryotic translation initiation factor 3 subunit 4	Transcription related	Transcription & Translation	-1.60	-2.36	-2.13
PSOLI00800	Nodulin26-like major intrinsic protein	Transport variety of uncharged solutes	Transport & Protein processing	-1.69	-2.13	0.01
PSOLI02068	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	-2.34	-3.12	-0.23
Cluster J						
PSOLI00625	Lectin-like receptor kinase 7	Protein amino acid phosphorylation	Metabolism	-1.24	-1.53	-0.65
PSOLI03966	Tubulin alpha-2/alpha-4 chain	Cell proliferation	Maturation Growth & Development	-0.81	-1.22	-0.16
PSOLI04829	Pisum sativum mRNA for putative glycine rich protein	Cell differentiation, pistil senescence and osmotic stress	Maturation Growth & Development	-1.34	-0.75	-0.11
PSOLI04552	Cysteine proteinase	Cysteine-type endopeptidase activity	Maturation Growth & Development	0.27	-1.10	0.11
PSOLI05008	Chlorophyll A-B binding protein	Photosynthesis	Metabolism	-0.79	-1.61	-0.15
PSOLI05007	Chlorophyll A-B binding protein	Photosynthesis	Metabolism	-0.83	-1.36	-0.14
PSOLI03095	Phosphoglyceromutase	Glycolysis	Metabolism	-1.23	-1.24	-0.02
PSOLI04820	2,3-bisphosphoglycerate mutase	Glycolysis	Metabolism	-1.03	-1.13	-0.48
PSOLI02775	Enoyl CoA hydratase-like protein	Fatty acid metabolism	Metabolism	0.00	-1.82	0.10
PSOLI00939	3-ketoacyl-CoA synthase 5	Lipid metabolism	Metabolism	-0.28	-1.26	0.06
PSOLI03652	AT5g38200/MXA21_90	Glutamine metabolic process	Metabolism	0.60	-1.32	0.06
PSOLI02497	UDP-glucose 6-dehydrogenase	Respiration	Metabolism	0.18	-1.32	-0.28
PSOLI02389	Disease resistance res ponse/ dirigent-like protein	Disease resistance	Stress & pathogen related	-1.10	-0.86	0.06
PSOLI02869	Genome polyprotein	RNA binding	Transcription & Translation	-1.26	-1.31	0.11
PSOLI01899	Histone H2B	Transcription related	Transcription & Translation	-0.69	-1.14	0.10
PSOLI02276	Proton pyrophosphatase	Vacuolar proton-pyrophosphatase	Transport & Protein processing	-0.70	-1.10	-0.66
PSOLI02917	Probable exocyst complex component 4	Protein transport	Transport & Protein processing	-0.62	-1.63	0.28
PSOLI04891	Expressed protein	Unknown	Unknown	-1.02	-1.45	-0.13
PSOLI01943	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	-0.33	-1.09	0.09

Appendix 10. Continued

Cluster	Annotation	Function	Functional group	Expression level (M*)		
				14 DAF	21 DAF	28 DAF
Cluster K						
PSOLI04742	Putative BURP domain containing protein	Dehydration-responsive protein RD22 precursor	Maturation Growth & Development	-1.56	-0.24	-0.72
PSOLI00349	Trypsin protein inhibitor 1	Endopeptidase inhibitor activity	Metabolism	-1.26	-0.53	-1.08
PSOLI00421	ORNITHINE CARBAMOYLTRANSFERASE	Amino acid biosynthesis and salvage	Metabolism	-1.16	-0.26	-0.62
PSOLI00589	Abscisic acid and environmental stress inducible protein	Stress tolerance	Stress & pathogen related	-1.12	-0.02	-0.97
PSOLI05211	BURP domain-containing protein	Putative dehydration-responsive /Resistant specific protein	Stress & pathogen related	-1.12	-0.19	-0.79
PSOLI05212	BURP domain-containing protein	Putative dehydration-responsive /Resistant specific protein	Stress & pathogen related	-1.61	-0.35	-0.34
PSOLI05196	Putative chloroplast nucleoid DNA binding protein	Proteolysis	Transport & Protein processing	-1.08	-0.16	-0.76
PSOLI05215	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	-1.19	-0.25	-1.35
PSOLI05214	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	-1.11	-0.20	-1.23
PSOLI05216	Cylicin-1	Unknown	Unknown	-1.31	-0.14	-0.92
PSOLI05213	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	-1.44	-0.07	-0.98
PSOLI01244	Hypothetical protein predicted by Glimmer/Critica	Unknown	Unknown	-1.17	-0.33	-0.55
PSOLI01886	BURP domain-containing protein 4	Unknown	Unknown	-1.14	-0.21	-0.76

M*: The mean centered relative gene expression value (in log2 scale), Green color indicated the genes up regulated in CDC Striker and pink indicated the genes down regulated in CDC Striker seed coats.